PANNUS INVASION INTO CARTILAGE AND BONE IN RHEUMATOID ARTHRITIS

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ACADEMIC DISSERTATION

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"Hope is the Last to Die."
-Old Russian proverb

To The Boys

CONTENTS

1. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Konttinen YT, **Ainola M**, Valleala H, Ma J, Ida H, Mandelin J, Kinne RW, Santavirta S, Sorsa T, Lopez-Otin C and Takagi M. Analysis of 16 different matrix metalloproteinases (MMP-1 to MMP-20) in the synovial membrane: different profiles in trauma and rheumatoid arthritis. *Ann Rheum Dis.* 1999; **58(11)**:691-7.

II **Ainola M**, Mandelin J, Liljeström M, Li T-F, Hukkanen M and Konttinen YT. Pannus invasion and cartilage degradation in rheumatoid arthritis: involvement of MMP-3 and interleukin-1β. *Clin Exp Rheumatol*. 2005; **23(5)**:644-50.

III **Ainola M**, Mandelin J, Liljeström M, Konttinen YT and Salo J. Imbalanced expression of RANKL and osteoprotegerin mRNA in pannus tissue of rheumatoid arthritis. *Clin Exp Rheumatol*. 2008; **26(2)**:240-6.

IV **Ainola M**, Li T-F, Mandelin J, Hukkanen M, Choi SJ, Salo J and Konttinen YT. Involvement of ADAM8 in osteoclastogenesis and pathological bone destruction. *Ann Rheum Dis.* 2009 **68(3)**:427-34. Epub 2008 Apr 8.

V **Ainola M**, Valleala H, Nykänen P, Risteli J, Hanemaaijer R and Konttinen YT. Erosive arthritis in a patient with pycnodysostosis: An Experiment of Nature. *Arthritis Rheum.* 2008; **58(11)**:3394-401.

The publications are referred to in the text by their roman numerals.
In addition, some unpublished results are presented.

2. ABBREVIATIONS

ADAM	a disintegrin and metalloproteinase
ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
ATP	adenosine triphosphate
ATPase	ATP synthase
BCIP	bromochloroindolyl phosphate
BSA	bovine serum albumin
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
ctrl	control
CTx	C-terminal cross-linked telopeptide of type I collagen
DAB	diaminobenzidine
DAPI	diamidino-2-phenylindole
DIG	digoxigenin
DMARD	disease-modifying antirheumatic drug
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FSD	functional secretory domain
GM-CSF	granulocyte macrophage-colony stimulating factor
GFP	green fluorescent protein
GST	glutathione-S-transferase
HLA	human leukocyte antigen
ICTP	carboxyterminal cross-linked telopeptide of type I collagen
IFN	interferon
Ig	immunoglobulin
IL	interleukin
kD	kilodalton
LB	Luria-Bertani
M-CSF	macrophage-colony stimulating factor

MHC	major histocompatibility complex
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MT-MMP	membrane-type matrix metalloproteinase
NA	numerical aperture
NBT	nitroblue tetrazolium
NF-kappa B	nuclear factor kappa B
OA	osteoarthritis
OPG	osteoprotegerin
PBGD	porphobilinogen deaminase
PT	pannus tissue
RA	rheumatoid arthritis
RANKL	receptor activator of nuclear factor kappa B ligand
rh	recombinant human
RNase	ribonuclease
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	small interfering ribonucleic acid
ST	synovial tissue
TGF	transforming growth factor
Th	helper T cell
TNF	tumour necrosis factor
TIMP	tissue inhibitor of metalloproteinases
TRAF	tumor necrosis factor receptor-associated factors
TRAP	tartrate resistant acid phosphatase
UTP	uridine triphosphate
qRT-PCR	quantitative reverse transcriptase-polymerase chain reaction

3. ABSTRACT

Rheumatoid arthritis (RA) is a chronic inflammatory syndrome that causes polyarticular swelling, stiffness, pain and potential for structural damage of joints and functional disability. One of the characteristic features of RA is a symmetrical pattern of the joint involvement affecting usually multiple small diarthrodial joints of the hands and feet. Despite of intensive research, the aetiology of the disease has remained unclear. It is assumed that an unknown antigen reaches the synovial tissue, initiating a local immune response leading to synovitis. Continuous inflammation leads to synovial hyperplasia and pannus formation, which is a soft tissue expanding on cartilage and invading into it and the underlying bone matrix. The leading edge of pannus is composed of fibroblast- and macrophage-like cells, which produce proteinases able to cause destruction of articular cartilage. Co-operation of mesenchymal cells and macrophage-like progenitor cells leads to local formation of osteoclasts, which invade the subchondral bone using acid attack and acidic proteinases. Pannus invasion leads to bony erosions, which are more or less permanent signs of joint damage.

Proteolytic pathways play major roles in the development of tissue lesions in RA. The degradation of extracellular matrix proteins is an essential consequence of pannus formation and invasion. Hyaline articular cartilage, which covers the articulating ends of bones, is mainly composed of collagen II and proteoglycan, and lies on a subchondral bone plate mostly composed of collagen I, which all are substrates for several metalloproteinases and cathepsins. In RA, these proteinases are involved in the degradation of articular cartilage and demineralized subchondral bone.

The aim of the study was to analyze the mechanism of pannus expansion and invasion into cartilage and subchondral bone, especially involvement of pannus derived proteinases and factors involved in matrix degradation and osteoclastogenesis. The key molecules concerned in these processes were studied at protein and mRNA levels using immunohistochemical staining, Western blotting, ELISA, in situ hybridisation and quantitative RT-PCR in rheumatoid patient and osteoarthritic control patient samples, as also in cytokine-stimulated and non-stimulated fibroblast and monocyte cell cultures. Formation of multinuclear osteoclast-like cells from peripheral blood mononuclear cells and mouse monocyte/macrophages was studied and a potential fusion protein was over-expressed using gene transfer (transfection) or inhibited using gene silencing (short interfering RNA

technology). The role of one of the most interesting bone degradative enzymes, cathepsin K, was studied more closely by using a pycnodysostosis arthritis patient as a human cathepsin K "knock-out" arthritis model.

Matrix metalloproteinases (MMPs) form a large "neutral" proteolytic enzyme family, which upon release from intracellular to the extracellular space are therefore able to degrade extracellular matrix. We observed significant expression of MMP-3 in pannus tissue, especially when stimulated by IL-1β. It could be one of the key enzymes in RA and is likely to contribute to joint destruction by directly degrading cartilage. In contrast, TNF-α stimulated MMP-1 was hypothesized to be involved in pannus invasion in early RA, both in inflammation and in degradation of extracellular matrix. Both these MMPs activate other pro-proteinases in complex cascades leading to joint destruction. Interestingly, MMP-13 was not found in traumatic synovium tissue samples, but was expressed in RA. However the increased expression was more prominent in osteoarthritis than RA samples.

Bone resorption requires demineralization of bone matrix by osteoclast-derived hydrochlorid acid as the first step. Osteoclasts have naturally been implicated in bone destruction in RA and we showed that pannus fibroblasts express a receptor activator of nuclear factor kappa B ligand (RANKL) able to stimulate osteoclast differentiation in the bone-pannus junction. The relation between RANKL and its natural inhibitor, a decoy receptor osteoprotegerin (OPG), in pannus tissue is imbalanced in favour for osteoclastogenesis. Some proteins of a disintegrin and a metalloproteinase (ADAM) family have been implicated in cell fusion. A recombinant protein of ADAM8 has been shown to be capable to induce osteoclast-like multinuclear cell formation and activity, perhaps by fusion of mononuclear precursor cells via interactions mediated by its disintegrin and/or cysteine-rich domain. We found an increased expression of ADAM8 in the pannus-hard tissue junction. Cathepsin K, a lysosomal acidic cysteine endoproteinase, is secreted outside the cell, especially into acidic environment between osteoclast and bone, where it degrades demineralized bone collagen matrix. However, absence of this important enzyme did not prevent bone degradation as was shown in our pycnodysostosis patient, who also had erosive arthritis, indicating that other enzymes can take over the role of cathepsin K.

In this study we explored pannus tissue in RA and its capability to invade into rheumatoid cartilage and bone by stimulating degradative enzymes or proteins, which can stimulate the

formation of multinuclear osteoclasts by cell fusion. The capability of nature to bypass proteins and factors by replacing them with similar ones will be a clinical problem, when medication is turning towards more specific biological drugs. A better understanding of pannus tissue invasion into cartilage and subchondral bone may provide an opportunity to find specific targets to treat this destructive disease, which could be useful in prevention and treatment to slow down or inhibit the degradation of joint structures and impairment of joint function.

4. REVIEW OF THE LITERATURE

4.1 RA and joint destruction

4.1.1 Structure of the joint

The diarthroidal synovial joint is a mixture of highly specialized connective tissues of bone, hyaline cartilage, synovial tissue, ligaments, menisci, tendons and fibrous capsule. Although the cells responsible for the synthesis of tissues are derived from the same mesenchymal precursor cell line, the extracellular matrix in all connective tissues is different and depends on the cells and their function in these tissues. In diseases such as rheumatoid arthritis (RA) and osteoarthritis (OA) degradation of the tissue exceeds its synthesis, resulting in a net decrease in the amount of extracellular matrix finally leading to erosions and thinning of the degenerating cartilage, laxity and loss of stabilizing peri-articular tissues.

Bone

Skeleton is an organ system, which consists of mineralized osseous tissue, also called bone tissue, endosteum, periosteum, bone marrow, nerves, blood vessels, lymphatic vessels and cartilage. Bone is composed approximately to 70 % of inorganic, mainly mineral compound called hydroxyapatite, to 22 % of organic material, mainly collagen type I and to 8 % water. Morphologically there are two types of bone: porous trabecular bone, also known as spongy bone, and dense cortical bone, also known as compact bone.

The maintenance of normal bone mass depends on the balance between osteoclastic bone degradation and osteoblastic bone formation following it. Osteoclasts are formed by fusion from the monocyte/macrophage precursor cells into multinucleated giant cells, which express osteoclastic markers cathepsin K, tartrate-resistant acid phosphatase (TRAP) and calcitonin receptor (Sapp 1976, Baron *et al.* 1986, Schett 2007) (Figure 1) and able to resorb bone. Formation of osteoclasts requires the presence of macrophage colony-stimulating factor for macrophage precursors (M-CSF; Tanaka *et al.* 1993) and RANKL as the differentiation factor to promote fusion (Wong *et al.* 1997, Anderson *et al.* 1997, Yasuda *et al.* 1998). For some information of the adjunctive role of tumor necrosis factor-α (TNF-α) and interleukin-1beta (IL-1β), see 4.1.4. on cytokines below. RANKL is produced by synovial fibroblast,

bone marrow stromal cells, lymphocytes, vascular smooth muscle cells and mast cells. Both M-CSF and RANKL mediated interactions are necessary for osteoclastogenesis, because a lack of either one of these molecules is sufficient to block osteoclast formation. M-CSF acts through colony stimulating factor 1 receptor (c-fms) on the precursor cells and RANKL, a member of the TNF superfamily, acts through RANKL receptor RANK (Dougall *et al.* 1999). The regulation of osteoclast differentiation is also affected by osteoprotegerin (OPG), a RANK homolog or decoy receptor, which blocks the RANKL-RANK interaction (Simonet *et al.* 1997). OPG is a member of TNF receptor superfamily and expressed by synovial fibroblasts, osteoblasts and endothelial cells. Osteoclasts are found only close to mineralized tissue, the presence of which might operate as an additional local signal for final osteoclast differentiation. When activated, osteoclasts move to areas of microfractures in the bone by chemotaxis. Bone resorption starts when migrating osteoclasts adhere to the bone matrix, which leads to cytoskeletal reorganization and cell polarization (Väänänen *et al.* 2000).

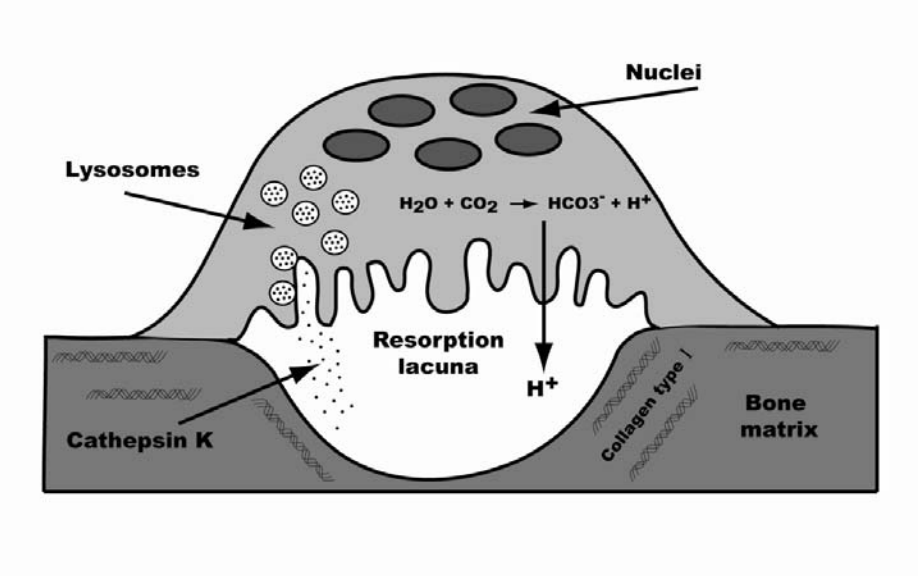

Figure 1. *Bone resorption by osteoclast occurs in two phases, first bone matrix is demineralized by local acidic environment and secondly, the collagen I-rich matrix is degraded by lysosomal secreted cathepsin K and other such acidic proteinases.*

Polarization creates four morphologically distinct areas of plasma membrane: i) the basolateral membrane, not connected to the bone matrix, ii) the tight sealing zone, where F-actin ring is in close indirect contact with the bone surface, iii) the ruffled border, a membrane area surrounded by sealing membrane facing the resorbing surface and iiii) a functional secretory domain (FSD) on the opposite site of the bone, a target for transcytotic

vesicles containing resorbed pieces of bone (Salo *et al.* 1996). After morphological differentiation to polarized cells, osteoclasts secrete protons by proton pump ATPase into the sealed extracellular compartment, the resorption lacunae, also called Howship's lacunae, to form an acidic environment, pH 3 or less (Baron *et al.* 1985, Silver *et al.* 1988 , Blair *et al.* 1989) for the dissolution of the bone minerals. This is followed by secretion of proteolytic enzymes for the degradation of exposed non-mineralized collagen matrix. The degraded bone matrix is either processed extracellularly or taken into the osteoclasts by endocytosis, followed by degradation within lysosomes (Salo *et al.* 1997). In addition to bone forming and resorbing cells, osteocytes are found embedded in the bone matrix located in their intercellular and -connected lacunar network and flattened bone lining cells on bone surfaces. Both of these cells are formed from mature osteoblasts. During development of RA, osteoclasts are formed locally from mononuclear precursor cells in the inflamed joint tissue and degrade the mineralized cartilage and subchondral bone (Gravallese *et al.* 1998).

Cartilage

Articular cartilage, also due to its somewhat glass-like visual appearance called hyaline cartilage, is found in diarthroidal joints covering articulating bone surfaces and absorbs the biomechanical impact and shear load upon movements of the gliding surfaces against another. Cartilage distributes the load and thus protects the subchondral bone from high stresses and reduces contact pressure. Cartilage provides a smooth, well lubricated surface for low-friction movement between joints. Cartilage structure is multilayered and identification of each layer is based on different characteristics like cell shape, morphology, cellular organization, collagen orientation and pericellular matrix deposition (Tyyni and Karlsson 2000) (Figure 2A). Cartilage is composed of water (70-80 %) and of solid phase, primarily of type II collagen (10-15 %), aggrecan (5-10 %) and various collagen decorating leucin-rich repeat protein molecules. Aggrecans are large proteoglycans, which fill the gaps of the collagen network by forming large aggregates interacting with hyaluronan and link proteins, and are highly hydrated. They provide the cartilage with a considerable swelling pressure and explain its ability to resist sudden compressive impact loads while water is seeping out from and later back to the matrix. Cartilage consists of lesser extent of collagen type IX and type XI and a relatively small number of chondrocytes, which are the main cells found in cartilage. Chondrocytes are located in lacunae and their function is to produce and maintain the cartilaginous matrix. Cartilage also contains a few mesenchymal stromal cells in the

superficial zone of the cartilage (Dowthwaite *et al.* 2004, van Osch *et al.* 2009). Collagen fibrils resist the swelling pressure of the proteoglycan matrix and give cartilage its tensile and shear strength. Alignment of collagen fibers give each zone particular biomechanical advantages which are provided by the arcade-like structure of the fibers: tangential, transitional and radial like orientations (Hunziker et al. 1997) (Figure 2B). A unique feature

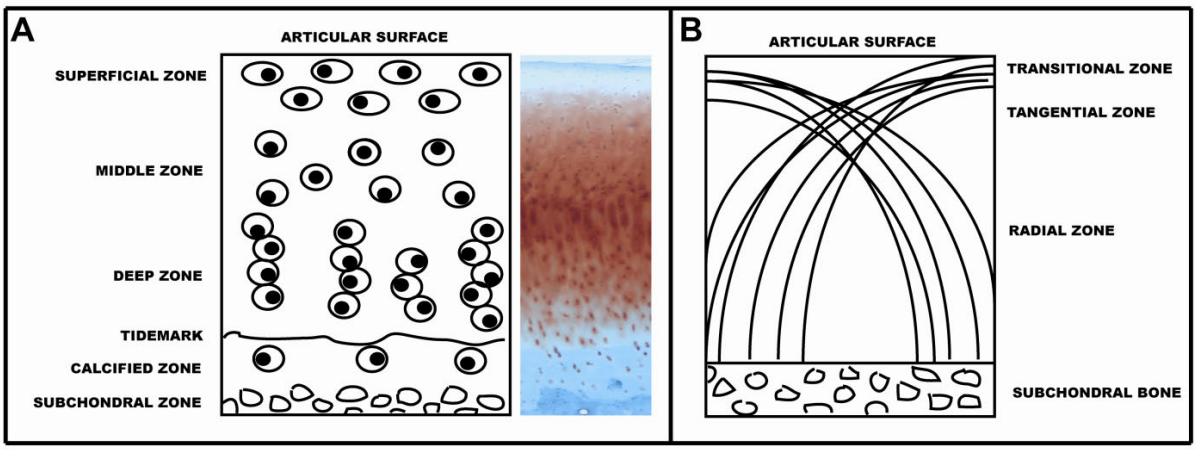

Figure 2. *The structure of articular cartilage. A) On the left side an illustration of the zonal architecture of articular cartilage. Superficial zone cells are small, elongated in shape, parallel to the joint surface; the middle zone cells are rounded and do not exhibit an organized orientation relative to the surface. Deep zone cells form groups of three or more cells arranged in columns perpendicular to the surface. On the right is a representative histological image with Safranin O staining from bovine articular cartilage of a knee joint. B) Schematic diagram of the cartilage matrix illustrating the arcade structure of the collagen fibres, starting off the subchondral bone and bending over as they approach the articular surface.*

of cartilage is the lack of blood vessels, lymphatics and nerves, which exposes it to externally controlled motor programs and the highly vascularized synovial tissue, which produces synovial fluid and provides thus nutrition for cartilage. In addtion, subchondral bone, which is a vascularized structure, also provides nutrients directly from the circulation to the deep zones of the cartilage. In RA, this articular hypoxic environment is changed due to neovascularization or angiogenesis, one of the characteristic features of this disease, which allows infiltration of inflammatory cells. The primary cause of cartilage degradation has been

suggested to be increased expression, strategic localization and enhanced activity of various proteolytic enzymes. In RA, degradation of cartilage is mediated by proteinases, derived from the inflamed synovium, activated synovial fluid granulocytes and stimulated chondrocytes. The loss of aggrecan is considered as an initial step during cartilage degradation, which is then followed by weakening of the collagen fibre network, followed by enzymatic and biomechanical degradation of collagen fibrils. In RA, degradation of unmineralized cartilage is suggested to be mediated, not by multinuclear osteoclasts or chondroclasts, but primarily by the synovial pannus tissue (Bromley and Woolley 1984). Furthermore, the close physical relationship between fibroblast-like and macrophage-like cells in the pannus also suggest that it has a potential to locally produce osteoclasts, which via hydrochlorid acid and cathepsin K mediate demineralization and collagen matrix degradation of articular bone.

4.1.2 Description of RA

The first recognized description of rheumatoid arthritis dates back to 1800 and was made by Augustin Jacob Landré-Beauvais in his thesis for his medical doctorate. RA is the most common inflammatory arthritis and despite of intense research, the etiology of the disease remains unclear, but it is interesting that compared to e.g. OA and ankylosing spondylitis, RA may be a relatively new disease. RA is associated with significant morbidity and increased mortality, and the prevalence rate of RA in different populations is 0.5-1 %, affecting women two to three times more often than men. Onset is uncommon in young people, the incidence rises with age and in Finland the mean age is approximately 59 years (Kaipiainen-Seppänen and Aho 2000). In Finland, the prevalence of RA is 0.8 %, approximately 32 000 suffer from this disease and 2000 adults get affected per year (Hakala and Kauppi 2007). The American College of Rheumatology 1987 revised criteria for the classification of RA are listed in table 1 and require at least 4 criteria to be present for at least 6 weeks (Arnett *et al.* 1988). RA is a heterogeneous syndrome, because it is defined by the presence of 4 or more criteria out of 7.

RA is not an inheritable disease, but close relationship raises the prevalence up to 2-4 % in first-degree relatives and dizygotic twins and up to 12-15 % in monozygotic twins (Lee and Weinblatt 2001). There is an inadequate knowledge of risk factors, but one of the most important predisposing environmental factors is smoking. Hormones or X chromosome-linked gene dose effects may affect the prevalence, which could explain the higher risk of RA in women. Increased disease activity after pregnancy, or termination of pregnancy, points to

the same direction. RA has also been suggested to be associated with joint injury and other environmental stimuli, like viruses, bacteria and stress, but this is uncertain. In contrast, certain major histocompatibility complex (MHC) class II genes and some other genes and regions are associated with RA and/or its severity. In conclusion, both genes and environment may contribute to the development of RA.

Table 1. *Classification criteria for rheumatoid arthritis as defined by The American College of Rheumatology in 1987.*

The American College of Rheumatology 1987 revised criteria
1) morning stiffness lasting at least 1 hour
2) joint swelling at least in three different areas
3) joint swelling in hand
4) symmetric joint swelling in the same area
5) rheumatoid nodules
6) rheumatoid factor in the blood
7) radiographic erosions and/or periarticular osteopenia

RA is considered as a chronic autoimmune disease, which causes the immune system to attack against substances or tissues normally present in the body. There is no specific diagnostic test available for RA. Different potential autoantigens are found in RA like circulating serum proteins, citrullinated proteins and peptides, nuclear components and components of articular cartilage. Rheumatoid factor, an autoantibody against the Fc portion of IgG, has traditionally been used in the diagnosis, but only 70 to 80% of RA patients have this factor in their serum and otherwise also normal healthy individuals can have it. Antibodies against citrullinated peptides have been shown to be more specific than rheumatoid factor. Recently antibodies directed against citrullinated collagen type I and type II have been detected more often in RA patients than in control groups. RA affects primarily joints, but it also affects other organs and is considered a systemic disease. The reason for the preferential and symmetric involvement of the small joints is not known though neurogenic inflammation in densily innervated small joints of the hands and feet has been suggested to

play a role here (Konttinen *et al.* 1994, Kidd *et al.* 1989). Trauma might have some localizing effect, so called deep Köbner's phenomenon, for example, rheumatoid nodules typically arise in places of the body subjected to pressure, e.g. on the extensor side of the ulnar bones. RA affects multiple synovial joints in a symmetrical fashion, being thus a polyarthritis with midline symmetry suggesting that cross-spinal reflexes rather than circulating cytokines play a role in the pattern of joint localization. Synchondrosis, synostosis or enthesis are rarely affected. ACR criteria refer to occurrence of synovitis in at least three different joint areas, although officially involvement of four joints would actually still be named oligoarthritis. As already mentioned, involvement of the small joints of the hands and feet is typical, although larger joints, like the shoulder and knee joints can be involved. The inflammatorily affected synovial tissue, which lines the non-cartilaginous surfaces of the interior joint lining, is vascular soft connective tissue with type I and III collagens as its key extracellular matrix (ECM) components. This tissue is organised so that vascularised sublining and fibrotic or adipose connective tissue stroma supports a lining or membrane comprised of two distinct cell types. Self renewing fibroblast-like type B synoviocytes (Konttinen *et al.* 1989) produce intercellular matrix molecules, cementing the lining cells together to a relatively coherent structure, and also secrete high molecular weight hyaluronan to increase the viscosity of the synovial fluid. Macrophage-like type A synoviocytes, the pregenitors of which are recruited from the circulation, clear the apoptotic neutrophils and other cells from the joint cavity (Müller-Ladner *et al.* 2005). The microcirculation of the synovial lining of joints is facilitated by a high density of fenestrated capillaries situated very close to the synovial surface and oriented toward the joint cavity. This orientation and intercellular wide gaps of the surface allow rapid exchange of fluid. Inflammatory mediators increase the permeability of the capillary cell walls by increasing the size of the pores, which increases leakage of fluid to inflammed tissue, contributing to swelling of the joint (Levic 1981). Bleeding may also occur from ruptured capillaries. Synovial fluid contains ultrafiltrated components of plasma, lubricin synthesized by superficial zone chondrocytes and synovial stromal cells and hyaluronic acid synthesized by synovial lining, which act as nutrients and lubricants for the joint cartilage. Type B lining cells contain lamellar bodies or phospholip scrolls (Dobbie *et al.* 1995), which are not seen in routine slides, because the fat solvents used in sample processing dissolve them way, but which may provide various phospholipis to promote contact point lubrication, perhaps with the help of surfactant proteins. The synovial lining is normally only a few cell layers thick, however, in RA there is an extensive increase in the number of cells in the lining layer, which becomes several layers thick and forms villi

because in particular the macrophage-like type A synoviocytes increase in numbers. Sublining layer becomes infiltrated with inflammatory mononuclear cells, including lymphocytes, macrophages and mast cells. Neutrophils increase somewhat in numbers in RA synovitis tissue, but they transmigrate rapidly from the intravascular compartment via the synovium to synovial fluid in the joint cavity, where they survive only 1-3 days, but due to their rapid turnover still form the major cell type in there. All these features contribute to the clinically typical joint swelling. All of these cells, including resident fibroblasts, produce cytokines, which together with locally produced autoantibodies, such as rheumatoid factor and anti citrullinated peptide antibodies, and immune complexes and complement, maintain the chronic inflammation in RA tissue. Also extraneous factors, like dietary intake of N-glycolylneuraminic acid (Neu5Gc), particularly from red meat and milk products, have been associated with chronic inflammation. As a result of inflammation, hyaluronan is increasingly locally synthesized and partly depolymerised leading to morning stiffness (Saari *et al.* 1991), at the same time as molecules which sensitize the primary afferent nociceptive nerves lead to tenderness on movement and pressure, or even pain at rest (Konttinen *et al.* 1994). A short and self-limited activation of the immune system has no clinically significant effect on bone, whereas prolonged immune activation is the main contributor to juxta-articular, erosive and systemic bone loss, which together with the increased propensity to fall lead to increased fracture risk in patients with chronic inflammatory rheumatic disease (Caetano-Lopes *et al.* 2009).

Continuous inflammation in synovium leads the membrane expansion, which forms pannus (Gr. A cloth) with an avascular leading edge containing fibroblast-like and macrophage-like cells and vascular granulation tissue, which reach onto hyaline articular cartilage and subchondral bone degrading and invading them. This leads to the formation of the typical erosions (Kobayashi and Ziff 1975, Shiozawa and Ziff 1983, Shiozawa *et al.* 1983) (Figure 3). Synovial expansion in RA is also referred as malignant mesenchymal transformation (Fassbender and Gay 1988) and a recent study confirms that the activation and destruction in RA uses similar pathways as are observed in progression of malignant diseases (Senolt *et al.* 2006). Pannus can also degrade cartilage from other side by invading first to underlying bone tissue and thus create a bidirectional attack (Goldring 2002).

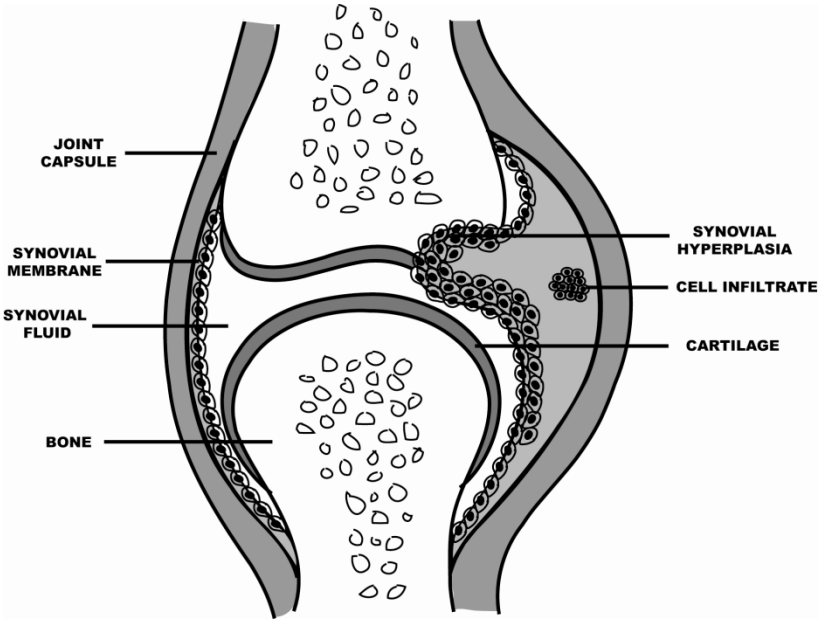

Figure 3. *A schematic presentation of a normal (left) and arthritic (right) joint. Pannus invasion into cartilage and subchondral bone is illustrated as synovial hyperplasia*

The leading edge of pannus is composed of a cell layer of fibroblast- and macrophage-like cells, which can produce proteinases, receptor activator of nuclear factor kappa B ligand (RANKL) and other factors causing directly or indirectly destruction of cartilage and bone matrices (Kinne *et al.* 2007, Muller-Ladner *et al.* 2007). During pannus invasion, the cartilage is covered with several layers of fibroblast-like cells, while invasion of macrophage-like cells occurs behind and beneath this layer (Shiozawa *et al*. 1983). Three types of pannus in cartilage-pannus junction have been observed: 1) cellular pannus without proliferating vessels, rich in phagocytic and fibroblastic cell, invaded in the hard tissues, 2) cellular pannus with proliferating small blood vessels, penetrated into the hard tissues, and 3) inactive fibrous pannus overlaying the hard tissues (Kobayashi and Ziff 1975). Two different histological features of cartilage-pannus junction have been observed, a distinct invasive cartilage-pannus junction, where a distinct junction is defined lying immediately adjacent to the cartilage surface; and a diffuse fibroblastic cartilage-pannus junction, also known as transitional fibroblastic zone, where the soft-hard tissue junction forms an indistinct cartilage-pannus junction (Allard *et al.* 1988). Eventually, synovitis leads to erosion of the joint surface, contributing to joint deformity, laxity and loss of function.

Medications to treat rheumatoid arthritis are used to relieve or reduce pain, reduce joint inflammations like swelling and tenderness, prevent or delay joint damage and deformity, improve daily function, prevent permanent disability and improve the quality of life. Apart from symptomatic non-selective and COX-2 selective non-steroidal anti-inflammatory drugs, medicines called disease-modifying antirheumatic drugs (DMARDs) decrease pain and inflammation, but also reduce or prevent joint damage. They are nowadays used early in the course of the disease and usually in changing evidence-based and empirical combinations with other such drugs. These immunomodulatory drugs are divided into two categories, non-biological DMARDs (eg. methotrexate, oxichloroquine, leflunomide) and biological DMARDs (eg. infliximab, etanercept, adalimumab, anakinra), which work in several different ways to suppress the patient's overactive immune and inflammatory systems (Lee and Weinblatt 2001, Feldmann and Maini 2008). The commencement of the DMARD effect may take over weeks or months ("slow-acting"), so nonsteroidal (eg. acetylsalicylic, ibuprofen) and steroidal (eg. glucocorticoids) anti-inflammatory drugs are used to provide faster relief of pain, stiffness, and swelling and maybe used also thereafter as adjunctive medication. Glucocorticoids have been traditionally classified as steroidal anti-inflammatory agents, but according to research data they also have slow acting, disease modifying action (Kirwan 1995, van Everdingen *et al.* 2002). Non-biological DMARDs are combined with each other or with biological DMARDs. Bisphosphonates, such as zolendronic acid diminish bone degradation by inhibiting osteoclast activity and expression of tissue degrading proteinases (Valleala *et al.* 2003). Bisphosphonates do not suppress inflammation, but are used successfully in combination with anti-rheumatic therapy to slow down destruction of bone and to prevent glucocorticosteroid mediated osteoporosis. The medical and occupational prognosis of the patients is better if the DMARD therapy is started early and targeted to achieve remission (Möttönen *et al*. 1999, Puolakka *et al*. 2004). .

4.1.3 Cell population in synovitis

Various cell populations, including monocytes/macrophages, B cells, T cells, endothelial cells, mast cells and fibroblasts are involved in the pathogenesis of RA (Firestein 2003). Two dominant cellular processes supposed to be operating in rheumatoid joint destruction are synovial lining hyperplasia and lymphocyte dependent immune responses (Figure 4A-C). Synovial hyperplasia is characterised by proliferating synovial fibroblasts and recruitment of infiltrating macrophages. Pannus can be envisioned as an extension of the synovial lining

attaching to and growing along the cartilage surface and into the subchondral bone (Figure 4D and E). Synovial fibroblasts originate from mesenchymal stem cells but are under normal homeostasis mainly maintained as a result of local proliferation. The role of type B synovial fibroblasts-like cells is to provide intercellular matrix molecules, such as type IV collagen (Poduval *et al.* 2007) and laminins (Konttinen *et al*. 1999a), to glue the lining cells to each other, and hyaluronan, lubricin and surfactant proteins for export for joint lubrication into joint cavity and adjacent cartilage. Type B synoviocytes are also involved in matrix remodelling by producing matrix degrading enzymes. The activated fibroblast phenotype

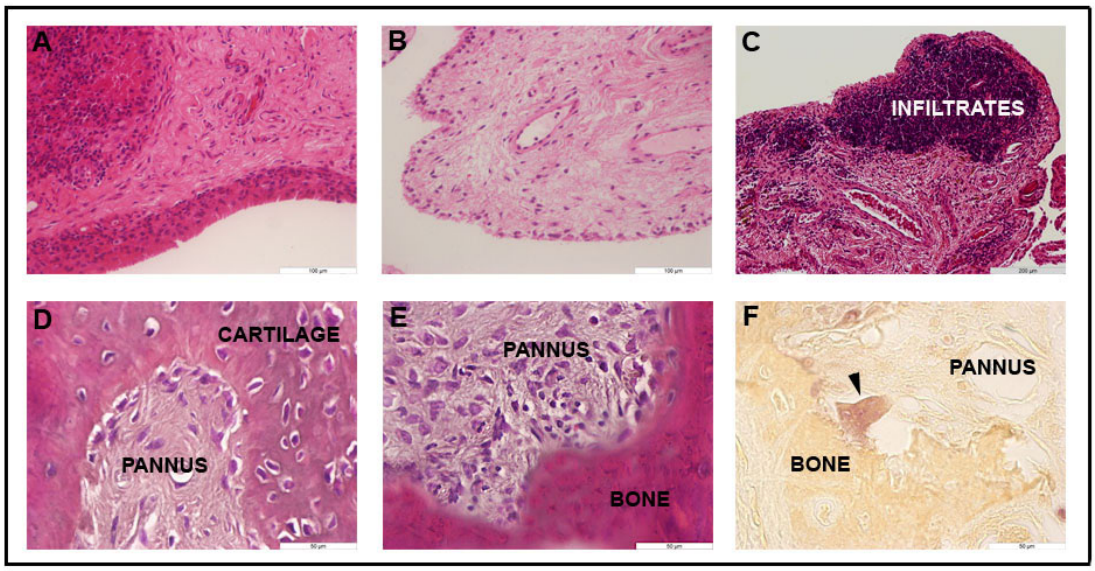

Figure 4. *A) Synovial lining hyperplasia in RA. B) Synovial lining in OA. C) Macrophage and lymphocyte infiltrates in RA. D) Pannus invasion into cartilage in RA. E) Pannus invasion into bone in RA. F) Multinuclear osteoclast (arrowhead) in the bone-pannus junction. Cell nuclei and proteins were stained using hematoxylin-eosin stain (A-E) and multinuclear osteoclast using TRAP stain (F).*

comprises changes in growth and expression, as well as responses to various stimuli. In early RA, synovial fibroblasts are probably activated already by innate immune system leading to production of inflammatory cytokines for attraction of inflammatory cells to the synovium and matrix destructive proteins for early perpetuation into surrounding tissues. In advanced RA, synovial fibroblasts are the main source of destructive molecules during pannus invasion, and the best known molecules involved in the destruction of the cartilage and bone are MMPs and cathepsins (Konttinen *et al*. 2000a, Rengel *et al.* 2007).

Monocyte/macrophages, as also osteoclasts and dendritic cells involved in RA (Figure 4F), are originated from myeloid progenitor of hematopoetic stem cells. These cells are not only activated and increased in synovial membrane and pannus-hard tissue junction for local response, but are also activated in peripheral blood and subendothelial space to mediate systemic responses. The increased filtration of macrophages correlates with the radiological progression of joint destruction (Mulherin *et al.* 1996). In RA and in inflammation, macrophages are activated by soluble stimuli or by cell-cell contact to produce pro-inflammatory effectors and tissue-degrading enzymes but also regulatory proteins, and participate both in the initiation and perpetuation of inflammation (Ma *et al.* 2003).

Development of RA may require triggering of the innate and/or the adaptive immune systems. Microorganisms, like bacteria and virus, will encounter the innate immune system so that these microbes are recognized based on their conserved pathogen-associated molecular patterns. In the adaptive immune system, which needs several days to develop a strong immune response and immunological memory, the B-cells and T-cells are affected by antigen which guides antibody and cytokine production to promote antigen removal. T-cells are considered to be central orchestrating components of the immune-mediated pathology in RA, which are of relevance not only for the immune system, but also regulate inflammation and tissue destruction. RA is described as an autoimmune disease, in which the immune system recognizes self-structures or autoantigens. It has been suggested that these autoantigens could in part be cartilage matrix collagen type II or some other cartilage specific and immunologically priviledged structure, which upon solubilisation or partial degradation can associate with the major histocompatibility complex (MHC), especially HLA-DR1 and HLA-DR4, on antigen presenting cells (Li *et al.* 2002, Brand *et al.* 2003). During the development of RA degradation of cartilage matrix may instead of dominant epitopes generate so called cryptic epitopes, which is envisioned to promote autoimmune responses and also to maintain it (Lanzavecchia 1995).

In RA, both the antibodies and the immune cells play a role in pathology. Disturbed immune T-cell homeostasis could contribute to joint pathology via impaired function of regulatory T cells and facilitation of pathogenic helper T-cells (Th cells). Studies of helper T cells originally led to the classification of RA as a Th1-like disease (Miossec and van den Berg 1997). This cell population, predominantly producing interferon-gamma (IFN-γ) and interleukin-2 (IL-2), sometimes in combination with low levels of IL-4 and IL-10, was found

to be dominant in RA synovium. In contrast, T cells predominantly producing IL-4 and IL-10 with Th2 cell phenotype were rarely found. One of the main effects of this Th1/Th2 imbalance is increased production of tumor necrosis factor-alpha (TNF-α), also a Th1-associated cytokine, which activates a cascade leading to chronic inflammation and destruction of hard tissues as can also be deduced based on the beneficial therapeutic effect of TNF-inhibitors in the treatment of RA (Elliot *et al.* 1994). Recently a novel Th cell subset producing IL-17 but not IFN-γ or IL-4, has been found and named Th17. Th17 pathway is involved in inflammation and these cells in mice are capable to enhance osteoclastogenesis (Sato *et al.* 2006).

4.1.4 Cytokines

Cytokines are low molecular weight proteins, acting locally as intercellular mediators and are important regulators in major biological processes. Cytokines are produced in quite small quantities, but usually have extensive effects, acting not only in direct events, but also subsequently in secondary events. This high efficiency is due to the significant avidity of cell-bound receptors and the ligand-induced activation of numerous potent intracellular signalling cascades. In RA, disturbance of immune system leads to the production and release of inflammatory mediators into the synovium and synovial fluid by infiltrating cells and resident synovial cells. Cytokines are considered to take part in each step of the development of RA disease from early autoimmunity step through continuous chronic inflammation into destruction of joint tissue. Numerous cytokines are expressed in synovial tissue and imbalance between pro- and anti-inflammatory cytokines induces chronic inflammation (McInnes and Schett 2007).

The inflamed synovium contain various macrophage- and fibroblast-derived cytokines that can support the activation of T-cells and *vice versa*. TNF-α and IL-1β, which have been widely studies in synovial tissue and in clinical studies, are considered as the main proinflammatory cytokines in the pathogenesis of RA (Feldmann *et al.* 1996, Maini *et al.* 1999, Bresnihan 2001). These cytokines are produced by synovial monocyte/macrophages, fibroblasts, lymphocytes and osteoblasts. Both TNF-α and IL-1β induce inflammation in arthritic joints and are also in part responsible for pannus invasion and the subsequent damage of bone and cartilage. TNF-α and IL-1β have a dual role in osteoclastogenesis, first by inducing RANKL expression and secondary, like TNF-α, by binding directly to osteoclast

precursor cells, and like IL-1β by modulating the expression of RANK in these cells and thus promoting osteoclast formation. TNF-α can independently stimulate osteoclast formation in the absence of the RANKL-RANK interaction, but in the presence of M-CSF (Kobayashi *et al.* 2000). Differentiation is mediated via TNF receptor on osteoclast precursor cells. IL-1 can directly stimulate osteoclast activation throught IL-1 receptor (Jimi *et al.* 1999) and this interaction is required for osteoclast function in TNF-α-stimulated precursor cell differentiation. Furthermore, TNF-α appears to influence the distribution of osteoclast precursor cells in the body by increasing their influx from the bone marrow into synovium. These cytokines participate in final steps of the degradation process by stimulating the synthesis of matrix degrading enzymes.

Cytokines are also modulating many animal models of arthritis. Inhibition of TNF-α suppresses various animal arthritis models, whereas the overexpression induces erosive inflammatory arthritis (Keffer *et al.* 1991). In animal models of arthritis, overexpression of IL-1 led to pannus formation and bone degradation (Ghivizzani *et al.* 1997), while blocking of IL-1 reduced disease activity and bone destruction (Joosten *et al.* 1999). Furthermore, therapeutic blockade of TNF-alpha suppresses clinical disease activity in 60-70% of patients with RA, while IL-1beta inhibition yields more moderate responses. On the other hand, both therapeutic approaches reduce or even prevent the extension of bone erosions (Maini and Taylor 2000).

Lately, more attention has been paid to IL-17, because IL-17 producing Th cells were found in synovial tissue (Chabaud *et al.* 1999). IL-17 refers to a group of cytokines that shares only a little homology with other cytokines. Six family members have been identified to date, which bind to a unique class of cytokine receptors. IL-17 is involved in pro-inflammatory activity as they can induce secretion of IL-6, IL-8 and GM-CSF (Gaffen *et al.* 2004). IL-17 can be also considered as a potent inducer of TNF-α, IL-1β and RANKL and, thus, does not only induce inflammation and cartilage degradation, but also osteoclastogenesis and bone resorption (Kotake *et al.* 1999, Jovanovic *et al.* 1998). IL-17 synergises with TNF, but also enhances inflammation and destruction independent of TNF-α and IL-1β (Koenders *et al.* 2006). IL-17 can promote joint inflammation and bone erosion, but not cartilage destruction (van den Berg *et al.* 2007). In arthritic mouse models, blocking of IL-17 prevents joint inflammation and bone erosion by decreasing RANKL and IL-1 (Koenders *et al.* 2005) and, conversely, overexpression of IL-17 worsens joint inflammation and bone damage (Lubberts

et al. 2002). Recently a new subpopulation of helper T cells, which produces IL-17 but not IFN-γ or IL-4, was shown in RA synovium and it is these cells which are now called Th17 cells (Aarvak *et al.* 1999, Yamada *et al.* 2008). However, these cells form only a minor subpopulation in RA synovitis tissue. It has been suggested, that sets of different cytokines and cells producing them are implicated in different stages of RA disease, which is supported by observations showing that IL-4 is expressed only in the early stage of the disease and IFN-γ expression varied depending on the state of RA (Dolhain *et al.* 1996, Raza *et al.* 2005).

4.2 Proteinases involved in tissue destruction in RA

Proteolytic pathways play major roles in the development of tissue lesions in RA. Degradation of extracellular matrix proteins is essential to pannus formation and invasion. Increased production of proteinases has been shown to be involved in many types of cancers and inflammatory diseases. Proteinases are responsible for hydrolytic cleavage of peptide bonds and are referred as exopeptidases or endopeptidases depending on the cleavage site (Figure 5) (Rengel *et al.* 2007). Exopeptidases can be subdivided into aminopeptidases with a

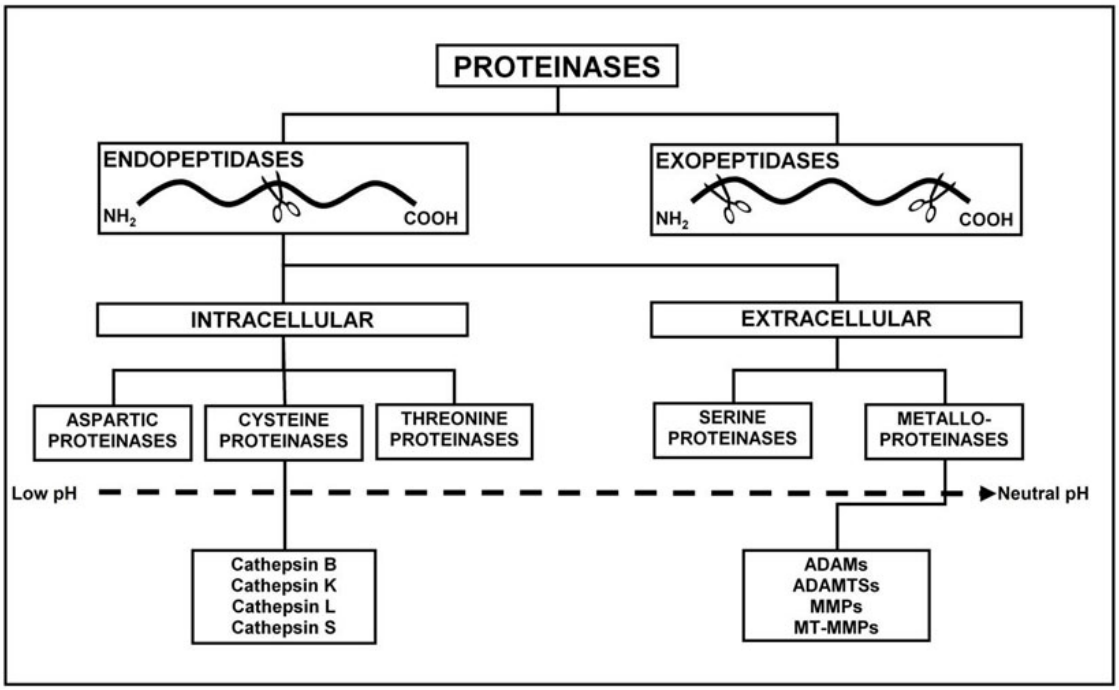

Figure 5. *Classification of proteinases based on the molecular site of action on the substrate, intra- and/or extracellular localization and pH optimum and structure of the active site.*

cleavage site at the aminoterminal end and carboxypeptidases with a cleavage site at the carboxyterminal end. Endopeptidase cleavage site is located in the middle of the substrate molecule. Such endopeptidases can be subdivided based on their catalytic mechanism reflected in the involvement of different chemical groups in their active sites. Aspartate, cysteine and threonine subtypes act usually intracellularly in acidic environment, whereas serine and metallo subtypes act extracellularly in neutral environment. Proteinases are involved in diverse biological processes such as digestion, blood coagulation, synthesis and activation of proteins, tissue remodelling in e.g. bone and cartilage, immune functions, ontogeny and apoptosis (Chang and Werb 2001). Many studies have been made concerning proteinases and their potential role via imbalanced regulation in arthritic diseases e.g. RA and OA.

4.2.1 Metalloproteinases

The metzincin superfamily belongs to zinc dependent metalloproteinases and is further divided into serralysins, astacins and in man important matrixins and adamalysins (Stocker and Bode 1995). The matrixins comprise MMPs. Adamalysins ADAMs and ADAMTSs are similar to the matrixins in their metalloproteinase domains, but also contain a unique disintegrin domain (Figure 6) (Duffy *et al.* 2003, Jones and Riley 2005). Various mechanisms are involved in the regulation of proteinases, including transcriptional and translational control, proenzyme activation, transportation and stabilization, inhibition or degradation of active enzymes. MMPs and ADAMs contain a prodomain with a “cysteine-switch”, which keeps the metalloproteinase site inactive (Nagase 1997). The active site contains a cysteine bound zinc atom, which is also coordinated to three conserved histidine residues. Upon release of the cysteine block, e.g. upon proteolytic removal of the activation pro-peptide containing the blocking cysteine residue, the active site of the enzyme is able to bind a water molecule necessary for the hydrolytic processing of the peptide bonds in the protein substrates. MMPs, ADAMTSs and many of ADAMs share this common zinc binding consensus sequence HEXGHXXGXXH followed by a methionine within the catalytic domain. In arthritic diseases, MMPs and adamalysins are considered to be the main enzymes responsible for degradation of aggrecans and collagens in cartilage, while some cathepsins and ADAMs seem to be involved in bone degradation.

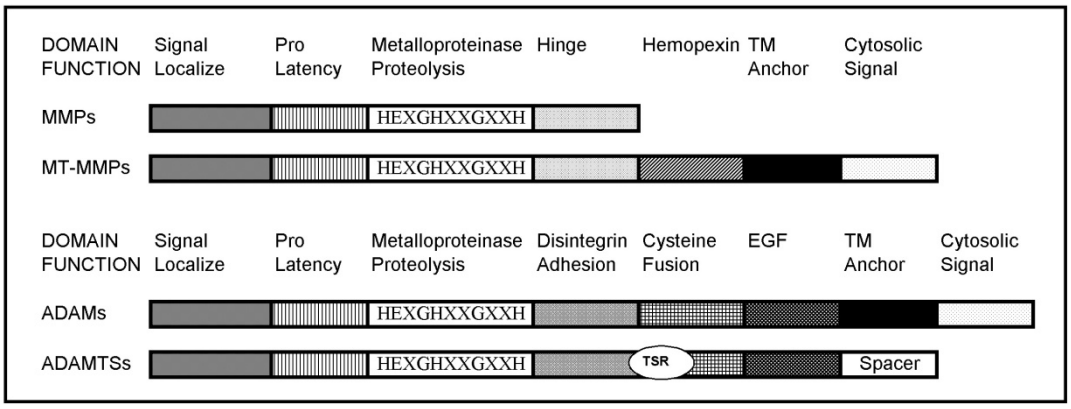

Figure 6. *Structure and function of matrixins and adamalysins. TM, transmembrane; EGF, epidermal growth factor; TSR, thrombospondin type 1 repeat.*

MMPs

MMP (matrix metalloproteinase) family proteins include both secreted and membrane bound proteinases with at least three shared domains (Figure 6), including the proteolytic domain involved in enzymatic digestion of the substrate(s). Wide varieties of tissues express MMPs, which are found in cells of both mesenchymal and hematopoietic origins, and these enzymes are involved in several physiologic and pathologic processes, including embryonic development, tissue morphogenesis, tumor invasion, cancer, angiogenesis, wound healing, and inflammatory diseases. Until now, 23 different MMPs have been identified, often divided into five subgroups, collagenases, gelatinases, stromelysins, matrilysins and membrane-type according to substrate specificity, structure and cellular localization (Martel-Pelletier *et al.* 2001). The MMP family members are best known for their ability to cleave components of the extracellular matrix, including the collagen, proteoglycan, fibronectin, and laminin, all of which are present in the connective tissues of the joints (Martel-Pelletier *et al.* 2001), but in addition to this they also can proteolytically activate other proteinases. Proteolysis often occurs in peri-cellular pockets close to the cell membrane, where localization of MMP concentrates enhances activity but limits the proteolysis to distinct pericellular regions. MMPs have some innate specificity for particular cleavage sites, but colocalization of the proteinases and their substrate also play a role in substrate selection (Turk *et al.* 2001). MMPs that have a furin recognition sequence are often activated already intracellulary within the Golgi complex and secreted in an active form, but most are secreted as inactive

proproteins which are activated extracellularly via cleavage by other extracellular proteinases or non-proteolytic release of the cysteine block. MMPs can be activated by serine proteinases, such as plasmin (Cawston and Wilson 2006), and also by each other (Murphy *et al.* 1987). All active MMPs are inhibited by tissue inhibitors of metalloproteinases (TIMPs) that bind tightly to active MMPs in a 1:1 ratio, but TIMPs can also considered as MMP carrier proteins or they may focalize some MMPs to a cell surface MT-MMP/TIMP complex.

Fibril-forming collagens are very resistant to most proteinases because of their rope-like triple-helical structure. Three collagenases, MMP-1, MMP-8, and MMP-13, but also MMP-2 and MT1-MMP, can degrade main type I, II and III fibrillar collagens at a specific initial cleavage site right across the triple helix at ^{775}Gly-^{776}Leu/Ile, which produces distinctive three-quarter- and one-quarter-sized degradation fragments. These collagenases, however, differ in their specificity for different collagen types. MMP-13 prefers type II collagen, whereas MMP-1 prefers type III and MMP-8 type I collagen. After the collagen monomer has been degraded, its helical structure is spontaneously in the body temperature unwound to a random coil, i.e. denatured into gelatin, which is further digested into smaller peptides by gelatinases, like MMP-2 and MMP-9. Stromelysins MMP-3 and MMP-10 can degrade proteoglycan core proteins, laminins, fibronectin, elastin, gelatin, and collagen types III, IV, V, VII, and IX. These two MMPs have similar substrate specificities, but different tissue localizations. Degradation of cartilage and bone matrix proteins by MMPs and cathepsins is the hallmark of synovial joint destruction. Many MMPs have been shown to be associated with RA and especially their involvement in cartilage destruction has been studied (Murphy *et al.* 2002). A beneficial role of some of the MMPs in the regulation of inflammation has been reported and their potential anti-inflammatory effect and protective role in RA has been emphasized (Gueders *et al.* 2005). A wide variety of MMP knock-out and transgenic mice models have been developed and lots of data, both for and against, have been gained of their role in development of arthritic lesions.

ADAMs

ADAMs (a disintegrin and metalloproteinases) also referred as MDCs (metalloproteinase disintegrin cysteine-rich proteins) are integral transmembrane proteins expressed primarily in monocyte/macrophages possessing both proteolytic and adhesive domains. Some ADAMs can also be spliced to form soluble proteins or, alternatively, can be shed from membrane to

form a soluble form. In mammals, many ADAMs are expressed in the testis and associated structures, while others, like ADAM8, ADAM9 and ADAM12, have more widespread somatic distribution. The presence of the multiple domains suggests involvement of ADAMs multiple functions, including proteolysis, adhesion, cell fusion and cell signalling (Stone *et al.* 1999). Nowadays up to 40 different ADAM peptidases are known and at least half of them contain the metalloprotease consensus sequence HEXGHXXGXXHD (Wolfsberg *et al.* 1995), which forms a zinc binding active site (Figure 6). ADAMs can also be classified as sheddases, because they cleave or shed extracellular parts of transmembrane proteins, e.g. cytokines and growth factors, with their metalloproteinase domain being also involved in degradation of extracellular matrix (Moss and Lambert 2002). The disintegrin domain is involved in adhesion events and interacts with integrins or other cell surface and extracellular matrix proteins (White 2003) to bring cells into close contact with other cells or matrix components but may also focus metalloproteinase domain into its site of action. Most ADAMs do not have conserved RGD integrin-binding consensus sequences so the associations then occurs in a RGD-independent manner (Eto *et al.* 2002). The cystein-rich domain in some ADAMs contains a putative fusion peptide, which has been proposed to be involved in at least two important cell-cell fusion processes, sperm-egg and myoblast fusion (Blobel *et al.* 1992, Yagami-Hiromasa *et al.* 1995). At the same time, the wide tissue distribution of ADAMs suggests participation also in other cell fusion events and, indeed, involvement in osteoclast formation has been reported (Abe *et al.* 1999, Namba *et al.* 2001). The cystein-rich domain is also implicated in adhesion and might cooperate with proteinase domain to control its function (Smith *et al.* 2002). ADAMs are usually activated by removal of the prodomain with furin or other proprotein convertases, but some seem to be also at least in part autocatalytically activated (Schlomann *et al.* 2002). Some ADAM proteins can be cleaved between pro-metallo sites to display metalloproteinase activity, followed by further cleavage between the metallo-disintegrin sites, which release the adhesion/fusion active disintegrin domains. Differences in the activity and subcellular localization depend on the ADAM, the cell type expressing it and substrates available. Some pathological conditions, such as inflammation, arthritis and cancer, have been shown to involve members of the ADAM family. In RA increased expression of cytokines, like TNF-α, has been shown and the release of this and other cytokines via shedding mediated by ADAMs may take part in the pathogenesis of the disease.

ADAM8 (CD156a, MS2) was originally cloned from mouse macrophages (Yoshida *et al.* 1990) and is expressed mainly in cells of the immune-inflammatory system, such as B cells, monocytes and granulocytes (Yoshiyama *et al.* 1997). ADAM8 is processed by autocatalysis into two different active forms. The processed form is produced by removal of the prodomain, whereas the remnant form is derived from it by a further removal of the metalloproteinase domain (Schlomann *et al.* 2002). Additional proteolytic cleavage between EGF and transmembrane domain results in two soluble forms, the complete ectodomain and the metalloproteinase domain. ADAMs are usually activated by furin-catalyzed removal of the prodomain, but for ADAM8 this step is dependent on a metalloproteinase (Schlomann *et al.* 2002), and occurs in part autocatalytically, but may need pre-processing of the prodomain (Hall *et al.* 2009a). The enzymatic activity of ADAM8 is not inhibited by tissue inhibitors of matrix metalloproteinases (TIMPs), which is the case for nearly all other membrane-associated metalloproteinases (Amour *et al.* 2002). Expression of catalytically active ADAM8 is associated with increased invasive activity and extracellular matrix remodeling (Wildeboer *et al.* 2006) and may have an important role in bone morphogenesis (Choi *et al.* 2001), possibly via its adhesion and/or fusion effects mediated via integrin receptors on the forming osteoclasts (Rao *et al.* 2006). Increased ADAM8 expression has been shown in synovial-like interface tissue around loosened hip prostheses (Mandelin *et al.* 2003a). ADAM8 deficience in mice did not cause any major defects during development or adult survival and was not associated with any pathological phenotypes (Kelly *et al.* 2005).

ADAM9 and ADAM12 were first found to be expressed in neonatal muscle and bone and adult bone (Yagami-Hiromasa *et al*. 1995). Further studies showed that mice lacking ADAM9 developed normally and did not show any major pathological phenotypes (Weskamp *et al.* 2002), while ADAM12 deficient models suggest that it plays a role in adipogenesis and myogenesis (Kurisaki *et al.* 2003). Increased expressions of both ADAM9 and ADAM12 have been implicated in synovial-like interface membrane around aseptically loosened total hip replacement implants (Ma *et al.* 2005, 2006). Both of these proteins have been shown to function as fusion proteins during multinucleated giant cell and osteoclast formation (Abe *et al.* 1999, Namba *et al.* 2001) and ADAM12 also in myoblast formation (Yagami-Hiromasa *et al.* 1995), which might suggest an eventual role in diseases characterized by bone tissue destruction. Beside expression in monocyte/macrophages, they are also expressed in osteoblast, the most abundant cell type in bone (Harris *et al.* 1997, Mohan *et al.* 2002).

ADAMTS

Members of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family are secreted enzymes widely expressed in both healthy and pathologic connective tissues. The catalytic domains of various ADAMTS proteinases share a high degree of similarity and contain the zinc-binding consensus sequence HEXXHXXGXXH, in which the catalytic zinc is coordinated by the three histidine residues. Unlike ADAM proteins, ADAMTS proteinases contain a conserved thrombospondin type 1-like repeat, between the disintegrin-like and cysteine-rich domain, which can be involved in binding to extracellular matrix (Kuno *et al.* 1997; Figure 6). It appears that the zymogen form of ADAMTS enzymes resides intracellularly and that the enzyme is activated upon secretion by a furin-catalyzed process. Activation of ADAMTSs via a cysteine switch mechanism, which mediates activation of most MMPs, has not been proven. At least some of the ADAMTSs can be inhibited by TIMP-3. Many of ADAMTSs have been found to be expressed in cartilage (Kevorkian *et al.* 2004) and they influence several inflammatory processes and are involved in cartilage metabolism and pathology. ADAMTS proteinases were first described in mice (Kuno *et al.* 1997) and by now 19 different ADAMTSs have been identified, which are further divided into small subgroups based on their structural characteristics and activities, e.g. aggrecanases involved in cartilage aggregan degradation form one such subgroup (ADAMTS-1, -4, -5, -8 -9 -15 and -20). The degradation of the aggrecan molecules by aggrecanases might be an early event in articular cartilage degeneration (Nagase and Kashiwagi 2003) so that MMPs are later engaged to degrade collagen matrix, suggesting that aggrecan protects collagen fibrils from direct degradation by collagenases (Pratta *et al.* 2003). The expression of the entire ADAMTS subfamily has been investigated in normal and OA cartilage (Kevorkian *et al.* 2004) and results provide data suggesting that many members of the ADAMTS subfamily may have important roles in cartilage tissue homeostasis and pathology.

4.2.2 Cysteine proteinases

Cysteine cathepsins are a large family of proteolytic enzymes involved in various biological processes and are also associated with many pathological conditions. Significant differences in cathepsins expression levels, activities and ratios have been described between various tissues. Man has eleven members of this endoproteinase family, cathepsins B, C, F, H, K, L,

O, S, W, X and Z (Zavašnik-Bergant and Turk, 2007). Cathepsins are mainly intracellular enzymes expressed in lysosomes of the endocytic pathway and capable to degrade native collagens and other components of the extracellular matrix in phagosomes. They have homologous amino acid sequences and 3D structures and an active site comprised of cysteine, asparagine and histidine residues. To control enzymatic activity cathepsins are synthesized as inactive zymogens, which are activated by proteolytic removal of the N-terminal propeptide or activation peptide. Activation is carried out proteolytically by other active enzymes or proceeds autocatalytically (Brömme *et al.* 1996, McQueney *et al.* 1997). Their stability and activity is dependent on the acidic pH prevailing in lysosomes, Howship's lacunae under the osteoclasts and stomach. Cathepsin K is possibly the most significant proteolytic enzyme of osteoclasts in this family.

Cathepsin K

About forty years ago it was found that proteinases involved in the degradation of the organic bone matrix are lysosomal acidic hydrolases (Vaes 1968). The identity of this enzyme named cathepsin K was first later discovered, first in rabbit osteoclasts (Tezuka *et al.* 1994) and afterwards the corresponding human cDNA was cloned independently by several groups (Brömme 1998). Cathepsin K is also expressed extracellularly and is capable to degrade native fibrillar collagen, but also several other components of the extracellular matrix and is involved in bone remodeling. The activation of cathepsin K has been shown to occur intracellularly, before secretion into the extracellular matrix (Dodds *et al.* 2001). Classical mammalian collagenases of the MMPs family have an initial specific cleavage site at ^{775}Gly-^{776}Leu(Ile), but cathepsin K is capable to cleave the triple helical collagen domains at multiple sites a bit like bacterial collagenases (Garnero *et al.* 1998). Predominantly cathepsin K is expressed in skeleton, but lower levels are found in other tissues because it is also expressed by synovial fibroblasts, synovial macrophages, chondrocytes of the articular cartilage, osteoclasts and osteoblasts (Rantakokko *et al.* 1996, Hou *et al.* 2001, Mandelin *et al.* 2006, Salminen-Mankonen *et al.* 2007). The best known role of cathepsin K is its involvement in osteoclast-mediated resorption of the demineralized organic bone matrix. This is due to its high collagenolytic activity, especially against type I collagen (Brömme *et al.* 1996), and its location in sites with low pH found in the resorption lacunae of osteoclasts (Yamaza *et al.* 1998) (Figure 1), but also in the acidic interface membrane surrounding loosened hip prosthesis implants (Konttinen *et al.* 2001).

Much of our current understanding on the role of cathepsin K in bone destruction derive from the usage of various genetically manipulated mouse models (Gowen *et al.* 1999, Saftig *et al.* 2000, Morko *et al.* 2005, Li *et al.* 2006), in which an arthritis has been superimposed (Salminen-Mankonen *et al.* 2007). Further information derived from pathological conditions involving bone and cartilage turnover, such as osteoporosis, osteoarthritis, osteopetrosis, osteosclerosis and RA (Vasiljeva *et al.* 2007). The basic observations demonstrate accelerated bone turnover and reduced trabecular bone volume in conditions characterized by an increased cathepsin K expression, whereas targeted disruption of cathepsin K results in osteopetrotic conditions and bone fragility (Kiviranta *et al.* 2001). Cathepsin K as also cathepsins S, B and L levels are increased in synovial fluid and lining indicating a potential role in initiation and/or progression of RA (Reddy *et al.* 1995, Liao *et al.* 2004, Yasuda *et al.* 2005). Cathepsin L might also play a role in bone resorption (Iwata *et al.* 1997), but the role of other cysteine cathepsins, beside the cathepsin K, in pathological degradation of extracellular matrix remains unknown. The proof for the major role for cathepsin K in bone collagen degradation was provided by the discovery that deficiency of this enzyme causes a rare autosomal bone disorder called pycnodysostosis.

Cathepsin K deficiency

Pycnodysostosis is a rare genetic disease of the bone with the main characteristic features being a short stature and fingers, with slow but progressive deterioration and densification of bones with a tendency to fractures (Maroteaux and Lamy 1962). The name "pycnodysostosis" describes well this disease as formation of abnormally dense (pykno) bone. The precise frequency of pycnodysostosis has never been determined, but it is quite well known for the late 19th century French poster artist Henri de Toulouse-Lautrec (1864 – 1901), so much so that this disease is sometimes referred as Toulouse-Lautrec Syndrome. His diagnosis has been made retrospectively from old photographs because his size, body proportions and health problems fit well with the characteristics of pycnodysostosis.

Pycnodysostosis is an autosomal recessive disease, in which the gene is situated on one of the non-sex chromosomes and two copies, one from each parent, is needed for disease development. The chromosomal localization of the gene responsible for pycnodysostosis was first mapped in 1995 into chromosome region 1q21 and once the location was identified, the potential genes were evaluated, and one of those was cathepsin K (Gelb *et al.* 1995). In 1996,

cathepsin K mutations were linked to pycnodysostosis, which was then concluded to be caused by cathepsin K deficiency (Gelb *et al.* 1996). The phenotypes of cathepsin K knock-out mice resemble those of the pycnodysostosis patients, although some differences have been reported (Kiviranta *et al.* 2005, Li *et al.* 2006).

5. AIMS OF THE STUDY

The main aim of this study was to understand the role of pannus tissue and involvement of its products in cartilage and bone degradation in RA.

1. Matrix metalloproteinases have been implicated in extracellular matrix degradation and RA is characterized by invasion of inflamed pannus tissue into cartilage and bone matrix. The first aim was to characterize all the known MMPs in RA tissue.

2. The second aim was focused to extend the previous one to some of those MMPs, which showed a potential role in RA and which were accordingly chosen for a more detailed analysis. The aim was to quantitate the expression of these MMPs in RA synovial and pannus tissues and to study their potential inducers.

3. Osteoclasts have been implicated in destruction of the subchondral bone in RA and the third aim was to characterize the eventual expression of receptor activator of nuclear factor kappa B ligand (RANKL), a factor essential for osteoclast differentiation, in pannus tissue.

4. To characterize ADAMs (a disintegrin and a metalloproteinases), newly identified metalloproteinases and potential osteoclast-activating factors for their eventual involvement in pannus tissue invasion and formation of osteoclast-like multinuclear cells by promoting fusion of mononuclear precursor cells.

5. The last aim was to study the role of the major osteoclast proteinase cathepsin K in pannus tissue and in cartilage/bone degradation. A cathepsin K deficient patient with pycnodysostosis, who had developed a chronic arthritis, was studied and used as “a human knock-out” arthritis model in functional analysis.

6. MATERIALS AND METHODS

6.1 Patients and samples

6.1.1 Tissue samples (I, II, III, IV)

The research plan was approved by the ethical committee of the Helsinki University Central Hospital. Guidelines of the Declaration of Helsinki were followed. RA patients fulfilled the revised criteria of the American College of Rheumatology (Arnett *et al.* 1988). Samples of RA synovitis tissue and pannus attached to cartilage/bone at sites of erosions were collected in parallel from patients undergoing total joint arthroplasty, synovectomy or arthroscopy. Control synovial tissue samples were obtained from traumatic patients undergoing arthroscopy or OA patients undergoing total hip arthroplasty. Traumatic injuries may induce a cascade of events which leads to local inflammation. Traumatic synovitis display more layers of lining cells and infiltrating inflammatory cells than healthy tissue, however, compared to RA, these findings were limited and inflammation after trauma is usually self-limiting and will resolve. OA was selected as one of the controls because it is characterized by cartilage degeneration and wear-and-tear, but relatively mild secondary synovitis and is thus distinguished from RA. None of the patients included in the present study had any clinical or microbiological signs of infection. The samples were during operation snap-frozen in dry ice precooled isopentane and stored at -70ºC until further processing.

6.1.2 Pycnodysostosis patient (V)

The patient was a 55 year old woman with short stature, only 143 cm, and had had several pathological bone fractures. Her right femur had altogether fractured for five times and left for four times. Her finger bones and the middle metatarsal bone were very short and had also undergone pathological fractures, which is a feature typical for pycnodysostosis. On the basis of these symptoms in 1976 the Clinic for Inherited Diseases diagnosed her to suffer from an autosomal inherited pycnodysostosis. In 1994 our patient developed arthritis. After remission in 1995, bone scanning confirmed arthritis in several joints and signs of several previous fractures. Because earlier psoriatic skin eruptions, her arthritis was considered psoriatic arthritis, characterized by erosions and ostelysis of the tips of the small bones, and anti-rheumatic medication was started. Recently also markers for rheumatoid arthritis and

inflammation have been detected; the main thing from the point of view of the current work, however, is that she has some form of chronic arthritis. Her arthritis continued to lead progressively to the development of bone erosions, acro-osteolysis and osteolysis of the phalanges in her joints and bones. Her parents, children or sisters do not suffer from this disease.

6.1.3 Cell culture

Fibroblasts extraction and stimulation (II, III)

Fibroblasts from RA and OA patients were established using explant culture method. Tissue samples were minced to small pieces and left over night in cell culture media containing 10 % fetal bovine serum (FBS) (BioWhittaker) and high 10% antibiotic concentrations (1000 U/ml penicillin and 1 mg/ml streptomycin) to elude microbe contamination. Next day the media was changed and the concentration of antibiotics was decreased to normal 1 % concentration. The media was changed twice a week. Fibroblast-like cells started to migrate out from the tissue explants and after approximately 2 weeks, when 60 % of the dish area was covered with monolayer of cells, the tissue pieces were removed and the cultures were either allowed to grow to confluence when used for analysis or split 1:6 using trypsin detachment for maintenance. Cells were characterized with immunofluorescence staining of fibronectin and vimentin and were used at passages 3-6. Cell numbers were counted using Z1 Coulter Particle Counter (Beckman Coulter) in a sample defined 17-27 μm window. For stimulations, cells were cultured to confluence in 6-well plates at $1x10^5$ cells per well. The cells were stimulated with different concentrations of recombinant human (rh) cytokines for various times: 1) TNF-α (R&D), 2) IL-1β (R&D) or 3) IL-17 (R&D). Two parallel wells were used for RNA extraction and one well with 4 coverslips for histochemical and immunohistochemical analysis.

Human monocyte isolation and stimulation (III, IV, V)

Human monocytes were isolated from freshly drawn peripheral blood using Ficoll-Paque density gradient (Pharmacia Biotech) to separate mononuclear cells from red blood cells and granulocytes. Cells were counted using Z1 Coulter Particle Counter (Beckman Coulter) in a sample defined 6-16 μm window. Cells were cultured in a 6-well plates at $1x10^6$-$1x10^7$ cells per well in 2 ml or in a 24-well plate at $5x10^5$-$1x10^6$ cells per well in 0.5 ml in MEM Alpha Medium with Glutamax-1 (Invitrogen) supplemented with 10 % FBS and 1 % antibiotics.

After one hour incubation the non-adherent cells containing predominantly lymphocytes were removed and the adherent monocyte/magrophage-enriched cultures, characterized by monocyte (CD14) and macrophage (CD68) markers, were further stimulated with different concentrations of human recombinant cytokines for various times: 1) TNF-α (R&D), 2) IL-1β (R&D), 3) IL-17 (R&D), 4) M-CSF (R&D), 5) RANKL (Alexis Biochemicals) or 6) a combination of IL-1β, M-CSF and RANKL. (III, IV, V)

Adherent monocytes/macrophages were also used for co-culture with fibroblasts, which were added together with 40 ng/ml rhM-CSF (R&D) and with or without different concentration of rhTNF-α, rhIL-1β, rhIL-17 (R&D) or rhRANKL (Alexis Biochemicals) for various times. (III)

Mouse monocyte/macrophage cell line (IV)
RAW 264.7 mouse monocyte/macrophages (TIB-71, ATCC) were cultured in high glucose DMEM (Gibco) with 10 % FCS and 1% antibiotics. Cells are refreshed twice a week and split 1:6 once a week using scraping without trypsination for detachment. Cells were cultured in 6-well plates at $1x10^5$ cells per well in 2 ml or in a 24-well plate at $5x10^4$ cells per well in 0.5 ml medium. Cells were counted using Z1 Coulter Particle Counter (Beckman Coulter) in a sample defined 8-14 μm window at passages 4-10.

Osteoclast formation and resorption (III, IV, V)
Human and mouse monocyte/macrophages were used to generate multinuclear osteoclast-like cells by fusion. Adherent human monocytes were stimulated with 1) rhM-CSF (25ng/ml, R&D), 2) RANKL (40 ng/ml, Alexis Biochemicals) and 3) transforming growth factor β1 (TGF-β1, 5ng/ml, R&D) to generate osteoclasts in 14 day cultures. Adherent RAW 264.7 mouse monocyte/macrophages were stimulated with glutathione-Sepharose column purified GST-RANKL (100 ng/ml) to induce osteoclasts formation in up to 9 day cultures. Multinuclear cells were stained for TRAP using leukocyte acid phosphatase kit (387-A, Sigma), which generates a dark purple colour.

The capability of the multinuclear cells to resorb bone was analyzed by culturing stimulated human and mouse monocyte/macrophages at $5x10^5$ and $5x10^4$ cells per well, respectively (as above), on dentine discs (Immunodiagnostic Systems Limited, IDS) in 96-well plates. Cells were stained for TRAP (as above) and after visualization brushed away for inspection of the

resorption pits formed on the dentin discs using toluidine blue staining (3 minute incubation in 1% Toluidine Blue, 1% Na-tetraborate x 10 H_2O –solution at room temperature). (V)

All cell culture work was done at least in triplicates.

6.2 RNA expression

6.2.1 RNA extraction and cDNA synthesis (I-V)

Total RNA from frozen tissue and cultured cells was isolated using TRIzol reagent (Invitrogen). Tissue samples were homogenized using a small blade in the Ultra-Turrax homogenizer (Janke & Kunkel, IKA-Labortechnik) until the tissue formed an even suspension and cells were lysed without homogenization in TRIzol solution. This mono-phasic solution of phenol and guanidine isothiocyanate maintains the integrity of the RNA, but disrupts cells and dissolves cellular components. Chloroform was added before centrifugation to separate RNA into the aqueous phase, from where it was recovered by precipitation with isopropyl alcohol. Intactness of the RNA isolates was controlled using ethidium bromide staining of 1% agarose gels after electrophoresis. After extraction of total RNA, mRNA was further isolated from this RNA solution using oligo$(dT)_{25}$ oligonucleotides covalently attached to magnetic polystyrene microbeads via 5' linker group (Dynal) to reduce the genomic DNA contamination and to improve the subsequent yield of specific cDNAs. The procedure relies on A-T base pairing, where oligo-dT oligonucleotides hybridize with the polyA tail of mRNA followed by separation of the mRNA containing beads with magnet. Messenger RNA extraction was controlled with spectrophotometric measurement. 50 or 100 ng aliquots of mRNA were reverse transcribed to cDNA with SuperScript™ Preamplification System (Invitrogen). It utilizes oligo(dT) for hybridization to the purified 3´ poly(A) tails of mRNA isolates, followed by first-strand cDNA synthesis with reverse transcriptase (RT) enzyme and removal of mRNA template with RNase H treatment. Extraction and transcription were controlled using housekeeping genes. Negative PCR controls were performed without RT enzyme or sample mRNA, whereas positive controls were performed using positive control RNA.

6.2.2 Design of primers and probes for PCR (I-V)

Gene sequences were searched from NCBI Entrez search system. Sense and antisense primers and PCR probes were designed using primer design software Primer3 (Rozen and Skaletsky 2000). Primers were usually 20 nucleotides and were designed to minimize primer dimers and complementary regions, not to contain over 3x nucleotide repeats, to have 45-55 % GC concentration and Tm 60°C. Probes were 26-30 bases long and were designed to have Tm 10°C higher than that of the primers, to contain the reporter dye on the 5' end and the quencher dye on the 3' end (Table 2).

Sequence similarity searches for right primer/template and probe sequences and template exon/intron breaks were done using NCBI blastn program. Primers and probes were produced by Oligomer (Helsinki, Finland) or Proligo (Paris, France). The identity of the product was verified by size after PCR amplification (Dynazyme, Finnzymes) and by sequence from 50 ng of isolated (QIAquick, Qiagen) amplicon using automated Applied Biosystems 373 A sequencer in Sequencing Core Facility in Haartman Institute (Helsinki, Finland).

For TaqMan-based technology, the primers were designed to be located inside one exon to allow the use of genomic DNA as a standard (III). Probes anneal to minus strand and had reporter dye FAM (6-carboxy-fluorescein) at the 5' end and quencher dye TAMRA (6-carboxy-tetramethyl-rhodamine) at the 3' end, which was also phosphorylated. For SYBR green (II, IV, V) the primer sets were designed to be located in different exons, with at least one intervening intron, to exclude contamination of the sample with genomic DNA.

Table 2. *Primers and probes used in RT-PCR, in situ hybridization and siRNA analyses. The genes of interest, their sequence accession numbers and the lengths (bp) of RT-PCR product are shown. Accession numbers are for the Entrez database.*

Name	Acc. No.	Sense	Antisense	bp
HUMAN				
β-actin	X00351	TCACCCACACTGTGCCCATCTACGA	CAGCGGAACCGCTCATTGCCAATGG	295
β-actin	X00351	CACCTTCTACAATGAGCTGC	AGGCAGCTCGTAGCTCTTCT	466
PBGD	X04217	ACATGCCCTGGAGAAGAATG	AGATGCGGGAACTTTCTCTG	237
MMP-1	X05231	CTGAAGGTGATGAAGCAGCC	AGTCCAAGAGAATGGCCGAG	428
MMP-2	J03210	GCGACAAGAAGTATGGCTTC	TGCCAAGGTCAATGTCAGGA	390
MMP-3	J03209	CTCACAGACCTGACTCGGTT	CACGCCTGAAGGAAGAGATG	294
MMP-7	Z11887	GTGGTCACCTACAGGATCGT	ACCATCCGTCCAGCGTTCAT	282
MMP-8	J05556	ATGGACCAACACCTCCGCAA	GTCAATTGCTTGGACGCTGC	532
MMP-9	J05070	CGCAGACATCGTCATCCAGT	GGATTGGCCTTGGAAGATGA	406
MMP-10	X07820	GTCCTTCGATGCCATCAGCA	CTTGCTCCATGGACTGGCTA	380
MMP-11	X57766	CAGGTGGCAGCCCATGAATT	GTACTGAGCACCTTGGAAGA	456
MMP-12	L23808	CCACTGCTTCTGGAGCTCTT	GCGTAGTCAACATCCTCACG	367
MMP-13	X75308	CTATGGTCCAGGAGATGAAG	AGAGTCTTGCCTGTATCCTC	390
MMP-14	D26512	CAACACTGCCTACGAGAGGA	GTTCTACCTTCAGCTTCTGG	380
MMP-15	Z48482	GCATCCAGAACTACACGGAG	TACCGTAGAGCTGCTGGATG	474
MMP-16	D50477	TGTACCTGACCAGACAAGAG	AGTGTCCATGGCTCATCTGA	384
MMP-17	X89576	GACCTGTTTGCAGTGGCTGT	ACGATCTTGTGGTCGCTGGT	473
MMP-19	X92521	CAGGCTCTCTATGGCAAGAA	GAGCTGCATCCAGGTTAGGT	397
MMP-20	Y12779	GACCAGACCACAATGAACGT	GTCCACTTCTCAGGATTGTC	374
ADAM8	XM_005675	CACAGAGGATGGCACTGCGTATGA	CGTGCACCTCAGTCAGCAGCTT	221
TRAP	M19534	CTGTCCTGGCTCAAGAAACA	CCATAGTGGAAGCGCAGATA	299
Cathepsin K	NM_000396	CAGTGAAGAGGTGGTTCAGA	CTTGCCTGTTGGGTTGTACA	406
Calcitonin-R	AY430048	CAAATGCTATGACCGAATGC	GTTGGACCAGGTTCGATTGT	232
For TaqMan				
β-actin	M10277	TCACCCACACTGTGCCCATCTACGA	CAGCGGAACCGCTCATTGCCAATGG	295
β-actin probe	M10277	mATGCCCTxCCCCATGCCATCCTGCGTp		
OPG	U94332	GGCATTCTTCAGGTTTGCTGTTCCTA	AGCTGTGTTGCCGTTTTATCCTCTCT	125
OPG probe	U94332	mATTTGCCTGGCACCAAAGTAAACGCAxp		
RANKL	AF019047	CCAACATTTGCTTTCGACATCATGAA	TGACCAATACTTGGTGCTTCCTCCT	143
RANKL probe	AF019047	mACGTCACTAAAACCAGCATCAAAATCCCAAxp		
MOUSE				
β-actin	X03672	CTTCTTTGCAGCTCCTTCGT	GTGCCAGATCTTCTCCATGT	310
ADAM8	NM_007403	AGCATGGAGAGCAGTGTGAC	CAGCTCCCATCAAAGCAGTA	274
TRAP	NM_007388	GATGACTTTGCCAGTCAGCA	ACATAGCCCACACCGTTCTC	275
Cathepsin K	BC046320	AGGGAAGCAAGCACTGGATA	AGCACCAACGAGAGGAGAAA	277
Calcitonin-R	NM_007588	TGCTATGACCGGATTCATCA	CAGCAATCGACAAGGAGTGA	322
Integrin β3	NM_016780	GACGCATCCCATTTGCTAGT	ACTGTGGTCCCAGGAATGAG	269
For silencing				
ADAM8 siRNA	NM_007403	GAACUCCAUUGGUGUAGCCTT	GGCUACACCAAUGGAGUUCTT	---
CTRL siRNA	NM_007403	CCAUAUCCGACUGGUGAUGTT	CAUCACCAGUCGGAUAUGGTT	---

6.2.3 Cloning for qPCR and design of in situ hybridization probes (II-V)

For quantitative PCR standard curve and probes for in situ hybridization a specific PCR product was inserted into plasmid and grown in bacterial culture to produce large quantities of target DNA. Briefly, the gene of interest was first amplified with specific primers and extracted from agarose gel. The PCR-amplified fragment containing 3' deoxyadenosine-overhangs was cloned to a topoisomerase I activated, linearized pCR®II TOPO vector with single, overhanging 3' deoxythymidine residues using TOPO TA Cloning® kit (Invitrogen). Cloned vectors were transformed into chemically competent *E.Coli* bacteria using heat shock and plasmids containing the insert were selected with X-gal white/blue screening on LB plates. Positively selected plasmids from blue colonies were grown overnight in Luria-Bertani (LB) media and isolated, followed by linearization of the constructs with restriction enzymes. The plasmids with insert were identified by gel electrophoresis after restriction enzyme digestions, with PCR amplification and sequenced from 1 µg of isolated DNA. The acquired sequence was verified with NCBI blastn program and the concentrations of the plasmids were analyzed spectrophotometrically.

For in situ probes, plasmid DNA was linearized using either Xho I or Hind III (New England Biolabs) restriction enzymes. DNA templates were ethanol precipitated and *in vitro* transcribed to either single-stranded antisense RNA probe using SP6 RNA polymerase or a sense RNA control probe using T7 RNA polymerase. Digoxigenin-conjugated UTP (DIG RNA Labeling Kit, Roche) was incorporated into the RNA probes during synthesis, which was followed by digestion of the DNA template with RNase-free DNase I. The orientation of the insert was determined by sequencing using M13 reverse and forward primers. (IV)

6.2.4 Conventional RT-PCR (I-V)

PCR amplification was performed using 1 µl (2.4 or 4.8 ng) of first strand cDNA (6.3.1), 0.4 mM of target specific primers (section 6.3.2), dNTP mix and 2U of the thermostable DNA polymerase (Finnzymes) or AmpliTaq Gold enzyme (Applied Biosystems) in 50 µl of PCR buffer. The reaction was run in a thermal cycler (RoboCycler 40 Temperature Cycler) for 40 cycles using one minute denaturation at +95°C, one minute annealing at the optimal temperature, one minute extension at +72°C and finally 10 minutes extra extension for the

last cycle. Amplifications without template, or without both template and primers, were performed as negative PCR controls. Amplified DNA was run on a 1% agarose gel and visualised with ethidium bromide under UV-light for size verification.

6.2.5 Quantitative RT-PCR (II-V)

Quantitative PCR amplification was performed using 2 µl (4.8 or 9.6 ng) of first strand cDNA, 0.25 mM of target specific primers (section 6.3.2) in LightCycler™ FastStart DNA Master Hybridization Probes or SYBR Green I PCR mix in LightCycler™ PCR machine (Roche). A serial 1:10 dilution of human genomic DNA for hybridization probes or cloned PCR fragments for SYBR Green were used to determine the mRNA copy numbers of the amplicons per housekeeping gene β-actin or porphobilinogen deaminase (PBDG). Copy numbers of the genes were calculated by the number of grams/molecules of the plasmid that contains the gene of interest. With SYBR green mix, the identity of the product was also verified by a melting curve analysis. Each individual sample was amplified at least two times for all genes of interest.

6.2.6 In situ hybridization (IV)

In situ hybridization was performed to localize and detect mRNA expression in tissue sections by hybridizing the labelled complementary RNA strand (antisense probe) to the sequence of interest. Frozen tissue sections (6 µm) were first fixed in acetone and after air drying refixed in 4 % formaldehyde. After washes the sections were incubated in acidic 10 mM sodium citrate, pH 6.0, at 80-90°C for 60 minutes for antigen retrieval, rinsed in buffer, and treated to block the polar groups using TEAA (0.1 M triethanol amine, pH 8.0, with 1:400 (v/v) acetic anhydride). Following dehydration in ethanol, sections were incubated in hybridization buffer (50 % deionized formamide, 10 % dextran sulfate, 1 x Denhardt's solution, 100 µg/ml yeast tRNA, 300 mM NaCl, 10 mM Tris-HCl, pH 7.6, 5 mM EDTA and 10 mM NaH_2PO_4) at 56°C for 60 minutes followed by incubation in hybridization buffer containing 1-2 ng/µl of digoxigenin-labeled antisense or control sense RNA probes at 56°C overnight in humid boxes. After posthybridization washes unbound single-stranded RNA was digested with RNase A. RNA hybrids were visualized using alkaline phosphatase-conjugated sheep anti-digoxigenin Fab (Roche), with the colour being developed in bromochloroindolyl

phosphate (BCIP), nitroblue tetrazolium (NBT) and levamisole followed by counterstaining in methylene green.

6.3 Protein expression

6.3.1 Histochemistry

Immunohistochemistry (II-IV)

Paraffin embedded tissue samples: Serial 3 μm thick sections were dried overnight and deparaffinizated. Staining was performed in DAKO TechMate^{TM} Automated Immunostainer 500 and processed at 22°C using DAKO ChemMate^{TM} Reagent System. The protocol followed MSIPE program with 30 minutes enzymatic digestion in 4 mg/ml pepsin for antigen retrieval and 1 hour incubation with primary antibody. The concentrations of the primary antibodies used were as follows: 10 μg/ml for mouse anti-human RANKL IgG_{2b} (R&D), 0.67 μg/ml for mouse anti-human MMP-1 $IgG_{2a/\kappa}$ (Chemicon) and 2.5 μg/ml for mouse anti-human MMP-3 $IgG_{1/\kappa}$ (Chemicon). The level of nonspecific immunoreactivity was determined using same concentration of isotype-matched antibodies derived from the species used to generate the primary antibodies. Endogenous peroxidase activity was blocked with 0.3% peroxidase solution. Staining was visualized using biotinylated secondary antibody against the IgG of the host species of the primary antibody and visualized using horseradish peroxidase-conjugated streptavidine, with H_2O_2 as substrate and diaminobenzidine (DAB) as dye. Nuclei were stained purple using hematoxylin counterstain. Hematoxylin and eosin stained was used for morphological evaluation of tissue samples, where intracellular and extracellular proteins are stained with eosin. (II, III)

Frozen tissue samples: 6 μm tissue sections were fixed in acetone and endogenous peroxidase activity was blocked with 0.3 % H_2O_2. The sections were blocked with normal goat serum (Vector Laboratories) and stained with a polyclonal rabbit antiserum recognizing cysteine/disintegrin domain of mouse ADAM8 (Choi *et al.* 2001), whereas the same concentration of nonimmune rabbit IgG (Dako) was used as negative control. Staining was visualized with biotinylated goat anti-rabbit IgG, followed by avidin-biotin-peroxidase complex. Peroxidise activity and nuclei were detected as with paraffin tissue sections. ADAM8 double staining with TRAP was done using TRAP staining leukocyte acid phosphatase kit (Sigma) after ADAM8 immunostaining. (IV)

Immunofluorescence (II-IV)

Tissue samples fixed with acetone or cell culture samples on coverslips fixed with 3 % paraformaldehyde were incubated in primary antibodies: 10 μg/ml mouse anti-human RANKL IgG_{2b} (R&D), 10 μg/ml mouse anti-human MMP-3 $IgG_{1/\kappa}$ (Chemicon), 10 μg/ml mouse anti-human ADAM8 IgG_{2B} recognizing metalloprotease-disintegrin domain (R&D Systems) or 5 μg/ml rabbit anti-human ADAM8 IgG recognizing catalytic domain (Triple Point Biologics). The level of nonspecific immunoreactivity was determined using the same concentration of isotype-matched antibodies of the host species of the primary antibody. Staining was visualized with fluorescent Alexa Fluor dyes 488 or 594-labeled secondary antibody against IgG of the host species of the primary antibody (Molecular Probes). Nuclei were stained with 4',6-Diamidino-2-phenylindole (DAPI) (Sigma). (II, III, IV)

In co-cultures of monocyte/macrophages with fibroblasts, the multinuclear osteoclast-like cells were stained first for TRAP with leukocyte acid phosphatase kit (Sigma) and nuclei were then stained with DAPI (Sigma). (III)

6.3.2 Western blots (II, IV)

Tissue samples were homogenized using the small blade of the Ultra-Turrax homogenizer (Janke & Kunkel, IKA-Labortechnik) in RIPA Buffer Set (Boehringer Mannheim), whereas cell culture samples were lysed in Passive Lysis buffer (Promega). Samples were ultrasonicated in Vibra-Cell 501 (Sonics & Materials), centrifuged and filtrated to remove cell debris. SDS-PAGE was performed using 10-75 μg total protein per sample, mixed with sodium dodecyl sulfate sample buffer and boiled for 5 minutes for protein denaturation and applied to gels. After electrophoresis, the gels were blotted onto nitrocellulose membrane (Bio-Rad Laboratories), blocked overnight in 3 % bovine serum albumin (BSA) and incubated in primary antibodies: 0.2 μg/ml polyclonal goat anti-human MMP-1 IgG (R&D Systems), 0.2 μg/ml polyclonal goat anti-human MMP-3 IgG (R&D Systems), 2 μg/ml monoclonal anti-human OPG IgG_{2a} (R&D), 0.2 μg/ml biotinylated polyclonal rabbit anti-human RANKL IgG (PeproTech) or 1 μg/ml polyclonal rabbit anti-human ADAM8 IgG recognizing the carboxyterminal end, all diluted in 2 % BSA / Tris-Buffered Saline. Detection was performed using alkaline phosphatase-conjugated secondary antibody against the IgG of the host species of the primary antibody (Jackson Immunoresearch Laboratories) or alkaline phosphatase-conjugated avidin (Sigma) with a colour development solution

(Alkaline Phosphatase Conjugate Substrate Kit), with BCIP as substrate and NBT as chromogen forming an insoluble black-purple precipitate.

6.3.3 Flow cytometry (IV)

Flow cytometry was used to identify and characterize subpopulations of cells by sorting with size, morphology and fluorescent label of markers on the surface of the cells. Cells were detached from culture plates and $1x10^5$ cells in 100 µl were incubated with 0.5 µg/ml monoclonal mouse anti-human monocyte marker CD14 IgG_{2a} (Dako), 4.5 µg/ml monoclonal mouse anti-human macrophage marker CD68 IgG_1 (Dako) or 10 µg/ml mouse anti-human ADAM8 IgG_{2B} recognizing metalloproteinase-disintegrin domain (R&D Systems), all diluted in 1% BSA. The level of nonspecific immunoreactivity was determined using same concentrations of isotype-matched IgG of host species of the primary antibodies. Cells were stained with fluorochrome fluorescein isothiocyanate (FITC)-conjugated secondary antibody against IgG of the host species of the primary antibody (Jackson ImmunoResearch Laboratories) diluted in 1 % BSA. The intensity of fluorescence was measured and analysed and the cells were analyzed by flow cytometry using FACScan analyzer with Cell Quest software (Becton Dickinson). The results are reported as the percentage of positive cells of all cells.

6.3.4 ELISA (II)

Cell culture media were centrifuged to remove cells and cell debris. Supernatants were diluted to the optimum working range and the amount of total MMP-3 (pro- and/or active) was measured using Human MMP-3 Quantikine ELISA Kit (R&D Systems), in which a polyclonal MMP-3 specific antibody has been used to pre-coat the wells of the microwell plate. MMP-3 present in supernatant and standard samples was bound by the immobilized antibody, and total amount of bound protein was detected with another enzyme-linked polyclonal MMP-3 specific antibody. The intensities of colour developed by substrate solution were measured at 450 nm using wavelength 540 nm for correction. All the samples and standards were measured in quadruplicates.

6.4 Functional assays

Inhibition (V)

In vitro analysis of bone resorption was performed also in the presence of a non-specific cathepsin K inhibitor E-64 (Sigma) to mimic the cathepsin K deficiency of the cells of the pycnodysostosis patient. E-64 is an irreversible, potent and highly selective cysteine protease inhibitor that does not affect cysteine residues in other enzymes. E-64 was selected because it is a specific cystein endoproteinase inhibitor, which permeates well into cells and tissues, has a low toxicity and is stable. The concentration of E-64 recommended by the manufacturer is 1-10 μM, but we used 0.5-1 μM concentrations because pilot tests showed successful formation of multinuclear cells in this range, compared to slightly inhibited formation using higher concentrations. Human monocyte/macrophages were cultured on dentine discs (section 6.1.3) in the presence of RANKL, M-CSF and E-64 for different times.

Transfection (IV)

Transfection was used to introduce a cloned ADAM8 DNA into mouse monocyte/macrophages for induction of ADAM8 expression. Mouse monocyte/macrophages were cultured as described (section 6.1.3) and transfection was performed 24 hours after GST-RANKL stimulation using 0.8 μg full length cDNA of mouse ADAM8 in pcDNA3.1/V-5 vector (a gift from Dr. Choi) and Lipofectamin™ 2000 (Invitrogen). Efficiency of transfection was verified using quantitative RT-PCR, expression of green fluorescent protein (GFP) and immunofluorescence staining of ADAM8. The effect of transfection on multinuclear cell formation was studied using TRAP staining (section 6.1.3).

Silencing (IV)

Post-transcriptional gene silencing was used to inhibit ADAM8 expression by using short RNA probes to base pair (hybridize) with target mRNA, which is under these circumstances degraded so that translation to active protein is prevented. Mouse monocyte/macrophages were cultured as described (section 6.1.3). Double-stranded ADAM8 and control siRNA with a 19-nucleotide complementary region and a 2-nucleotide TT 3' overhang at each end were designed and synthesized by Proligo (Paris, France; table 2) for siRNA silencing. Silencing was performed twice at 24-hour interval after GST-RANKL stimulation of RAW 264.7 using 80 pmol siRNA per well and Lipofectamin™ 2000 (Invitrogen) for transfection. Mismatch siRNA duplex and Lipofectamin™ alone without siRNA were used as controls. Efficacy of

ADAM8 silencing was verified using quantitative RT-PCR. The effect of silencing on multinuclear cell formation was studied using TRAP staining (section 6.1.3).

6.5 Image analysis and statistical analysis

Image analysis (II-V)

Tissue samples, cell cultures and dentine discs with resorption pits were inspected using Olympus AX70 microscope and images were acquired with a 12-bit CD camera (SensiCam). Images were analyzed using a semiautomatic AnalySis Pro 3.0 image analysis and processing software (Olympus). The depths of the resorption pits were determined using a 20x/ 0.70 numerical aperture (NA) objective for focusing a laser beam in a sample and a Leica TCS SP2 AOBS laser scanning confocal microscope on reflection mode (Leica Microsystems AG, Mannheim, Germany).

Statistical analysis (II-IV)

Statistical analysis was performed using GraphPad Prism version 3.02 for Windows (GraphPad Software). Paired t test was used for comparison of paired samples. One-way ANOVA and nonparametric methods were used for multiple group comparison combined with Tukey's test for all possible pairwise comparisons, comparing the mean of every group with the mean of every other group, Dunnett's test for all against a control group and Bonferroni's test for only a few comparisons based on our aims. All data are expressed as mean and SEM and $P < 0.05$ was considered to be significant.

6.6. Analysis in other laboratories (V)

Collagen degradation analysis

Cathepsin K generated bone degradation products (C-terminal cross-linked telopeptide of type I collagen = CTx) were analysed using an automatic Elecsys instrument (Roche) or an Crosslaps ELISA assay (Nordic Bioscience) and MMP- generated bone degradation products (type I collagen carboxyterminal cross-linked telopeptide = ICTP) were analysed with radioimmunoassay (Orion Diagnostica) in serum and cell culture samples. Analyses were done in Professor Juha Risteli's laboratory in the Department of Clinical Chemistry (Oulu University Hospital, Oulu, Finland) (Risteli *et al.* 1993).

Measurement of active cathepsin K

Active cathepsin K was measured in Dr. Roeland Hanemaaijer's laboratory in TNO Quality of Life (Biomedical Research, Leiden, the Netherlands) with modified urokinase assay (Lindeman *et al.* 2004). Plates were coated with cathepsin K-specific monoclonal antibody (TNO) recognizing native cathepsin K and incubated with cell culture supernatant samples for binding of cathepsin K. Activity was measured by incubation of the captured cathepsin K with a modified prourokinase variant (UKcatK) in which the endogenous plasmin activation site has been adapted to a cathepsin K-specific activation site. Activated UKcatK was quantified using a chromogenic peptide substrate (Biosource Europe) and colour development was recorded at 405 nm.

7. RESULTS AND DISCUSSION

Active inflammation in rheumatoid arthritis causes formation of inflamed tissue (synovitis tissue and pannus tissue) which expands and invades into articular cartilage and subchondral bone damaging both tissues. Growing pannus cells and tissue are capable of releasing proteinases, which promote inflammatory cell invasion and hard tissue destruction. In RA hard tissue destruction is thought to be driven by pannus tissue, but the damage to cartilage and bone is mediated by distinct patophysiological pathways. Fibroblast-like cells, in co-operation with macrophage-like cells, are capable of mediating proteolytic cartilage damage. Instead, multinuclear bone resorbing osteoclasts, which are found in rheumatoid erosions, can directly damage bone, but do not appear to affect cartilage.

7.1 Synovial inflammation and pannus formation (I, II)

MMPs are members of a large proteolytic enzyme family and degrade extracellular matrix. In early RA increased expression of MMPs in pannus participates in the invasion of cartilage by degrading extracellular matrix and by activating other proteinases. Increased expression of some MMPs was shown in RA tissue compared to trauma tissue samples in the first work of this thesis and some of these MMPs were analysed in more detail in the second thesis work.

In RA synovitis tissue the most exclusive expression was observed for MMP-13, which was not detected in synovial tissue of patients with trauma (Table 3). MMP-13 degrades type II collagen particularly well. It is expressed by chondrocytes and synovial cells in RA and localized in pannus-hard tissue junction (Lindy *et al.* 1997, Konttinen *et al.* 1999b). It has

Table 3. *mRNA expression on different MMPs in the traumatic (N=9) and rheumatoid synovial membrane (N=10) samples. The results of conventional RT-PCR are given as the number of positive samples per all samples.*

	MMP-1	MMP-2	MMP-3	MMP-7	MMP-8	MMP-9	MMP-10	MMP-11	MMP-12	MMP-13	MMP-14	MMP-15	MMP-16	MMP-17	MMP-19	MMP-20
RA	10/10	10/10	10/10	7/10	4/10	10/10	6/10	10/10	6/10	9/10	10/10	8/10	6/10	9/10	10/10	0/10
Trauma	6/9	9/9	8/9	3/9	3/9	5/9	1/9	9/9	3/9	0/9	7/9	0/9	7/9	3/9	8/9	0/9

been shown that overexpression of MMP-13 increases synovial inflammation, but MMP-13 was rarely associated with damage of the articular cartilage after intra-articular injection of MMP-13 coding adenovirus vector (Joronen *et al.* 2004), indicating that it may play a role during the early phases of arthritis. In the second thesis work quantitative mRNA data on MMP-13 showed perhaps a slight tendency to an increase in OA compared to RA, but this difference was not significant (Figure 7A). This is in line with the earlier finding that chondrocytes produce MMP-13 in OA (Murphy *et al.* 2002) and with recent observations about the key role of MMP-13 in the development of OA (Takaishi *et al.* 2008). MMP-13 has, however, been found to be highly expressed in destructive periodontal disease, suggesting a role in the periodontal ligament and alveolar bone loss developing in this disease (Hernandez *et al.* 2006), or in osteoblast-mediated bone matrix degradation (Parikka et al. 2005). MMPs have also been suggested to produce collagen fragments which facilitate recruitment and activation of osteoclasts. Expression levels of MMP-13 were lower than those of MMP-3, which was found in high levels particularly in pannus tissue (Figure 7B). In

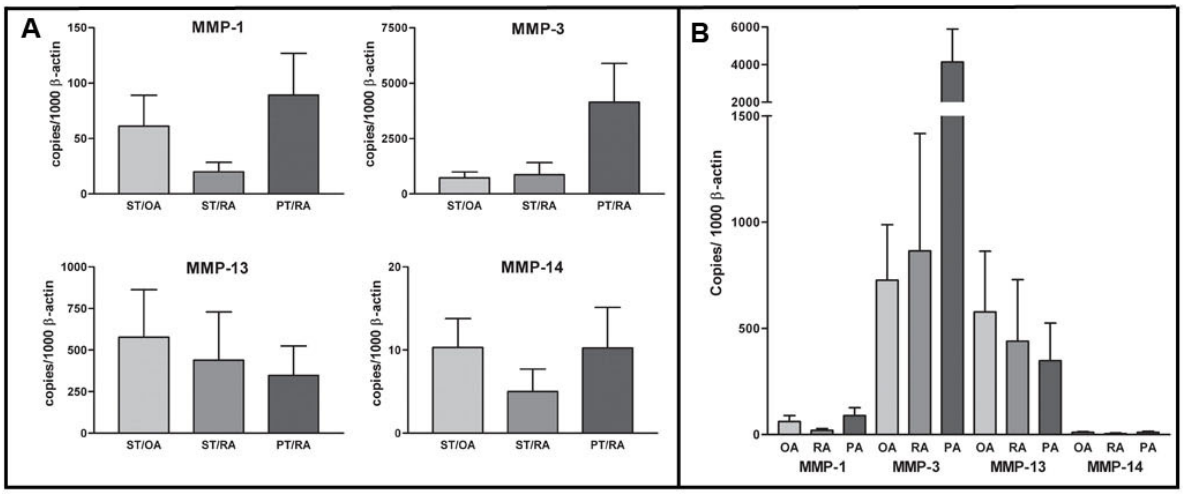

Figure 7. A) *Quantitative mRNA expressions of MMP-1, -3, -13 and -14, normalized for β-actin in osteoarthritic synovial tissue (ST/OA, n = 9), rheumatoid arthritic synovial tissue (ST/RA, n = 9) and pannus tissue (PT/RA, n = 8) in different panels. B) Messenger RNA expression levels of MMP-1, -3, -13 and -14 normalized for β-actin in osteoarthritic synovial tissue (ST/OA), rheumatoid arthritic synovial tissue (ST/RA) and pannus tissue (PT/RA) shown all in the same scale.*

OA the cartilage destruction is driven by chondrocytes, so also in RA MMP-13 might be involved with chondrocyte mediated cartilage destruction rather than invasion of pannus into the cartilage.

MMP-14 was expressed in all RA samples, but not in all control trauma samples (Table 3). Antisense MMP-14 treatment reduces the invasiveness of synovial fibroblasts and degradation of cartilage (Rutkauskaite *et al.* 2005). MMP-14 has been shown to be expressed in the invasive pannus tissue in RA and to be coexpressed in a complex with TIMP-2 and MMP-2 (Konttinen *et al*. 1998). It is produced by synovial fibroblasts but also by macrophages and osteoclasts. MMP-14 plays a role, not only in the fibroblast-mediated matrix degradation, but also in osteoclast-mediated bone resorption (Pap *et al.* 2000, Delaisse *et al.* 2003) and is the only MT-MMP expressed in chondrocytes. It can activate pro-MMP-2 and pro-MMP-13, which points out, that fibroblasts, macrophages and chondrocytes may co-operate in the degradation of cartilage and bone. In our analysis, mRNA expression levels of MMP-14 in OA and RA were very low (Figure 7B), but at a similar level (Figure 7A), suggesting perhaps a role in osteoclast and chondrocyte mediated rather than pannus driven cartilage tissue destruction. Miller *et al.* (2009) found high expression of MMP-14 at the cartilage-pannus junction in RA joints, which also points out the function of high and localized expression of factors at the exact site of degradation.

MMP-1 (interstitial collagenase) was the first detected MMP in rheumatoid synovium (Evanson *et al.* 1967). Previous studies have shown an important role for MMP-1 in cartilage collagen II degradation (Saito *et al.* 1998), while MMP-3 (stromelysin-1) is capable to degrade another cartilage matrix component, namely proteoglycan (Sapolsky *et al.* 1976). In our preliminary data MMP-1 was expressed in all RA and a few trauma samples, while MMP-3 was constitutively expressed in both conditions, but much more so in RA (Table 3). Increased expressions of MMP-1 and MMP-3 have been reported in rheumatoid synovial membrane (Hasty *et al.* 1990, Sorsa *et al.* 1992), in synovial fluid (Yoshihara *et al.* 2000) and in synovial cells (Okada *et al.* 1987), which correlates with invasive properties (Tolboom *et al.* 2002). MMP-3 acts as an indicator of disease activity and of the development of radiological damage in RA (Posthumus *et al.* 2000) and studies of MMP-3 knockout mice suggest an important role for it as a pro-MMP activator and in tissue degradation (van Meurs *et al.* 1999). MMP-1 levels seem to correlate with the degree of synovial inflammation (Maeda *et al.* 1995). Inhibition of MMP-1 reduces the invasiveness of RA synovial

fibroblasts (Rutkauskaite *et al.* 2004). Our conventional PCR data showed both MMP-1 and MMP-3 to be intensively expressed in RA samples, but with quantitative PCR a clear difference was observed in their expression levels. Both MMPs were increased in pannus tissue compared to synovial tissues (Figure 7A), but MMP-3 was found in pannus tissue in 46 times higher levels than MMP-1 (Figure 7B). In addition, MMP-1 had higher mRNA levels in OA than in RA synovium, while MMP-3 was particularly high in pannus tissue, which indicates an important role for MMP-3 in RA cartilage tissue destruction. MMP-1 and MMP-3 were also studied at protein level, which showed a similar pattern so that MMP-1 staining was weaker than MMP-3, but both showed increased staining in pannus compared to synovitis tissue (Figure 8). Unfortunately, it is not possible to compare staining intensities of

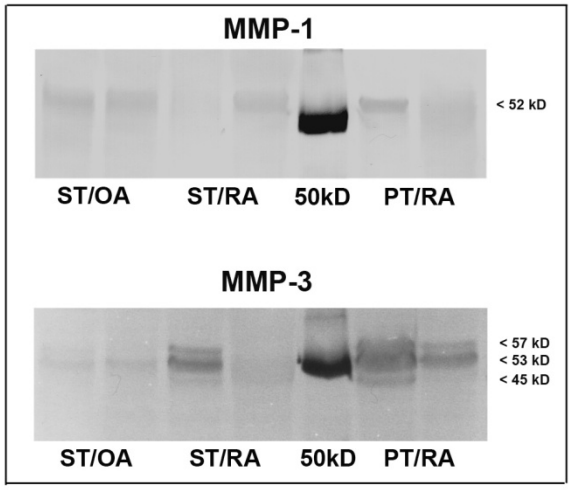

Figure 8. *Western blots showing latent 52 kD form of MMP-1, and latent 57 kD, intermediate 53 kD and active 45 kD forms of MMP-3 (together with a 50 kD molecular weight standard). Samples represent osteoarthritic synovial tissue (ST/OA, n = 2), rheumatoid arthritic synovial tissue (ST/RA, n = 2) and pannus tissue (PT/RA, n = 2), two lanes each.*

MMP-1 and MMP-3, because two different antibodies were used and MMP-1 antibody might due to its inherent properties give weaker staining than MMP-3 antibody. However, expression of the active 45 kD form of MMP-3 was clearly seen in pannus tissue extracts but this form of MMP-3 was weak in synovial tissue and was not expressed in OA synovium at all, while no active 43 kD form of MMP-1 was seen in any of the samples studied. Involvement of MMP-1 and MMP-3 in pannus tissue invasion has been indirectly supported

by a study dealing with the metastasis-associated protein S100A4, which promotes remodelling of the extracellular matrix and thus promotes progression of cancer and tumor. It was shown to be expressed in RA and to upregulate both of these MMPs (Senolt *et al.* 2006). Extracellular matrix metalloproteinase inducing protein EMMPRIN is similarly increased in RA synovial membrane (Konttinen *et al.* 2000b).

Based on these results and knowing that MMPs in general are upregulated by IL-1β and TNF-α, the effects of these cytokines on MMP-1 and MMP-3 in cultured synovial fibroblasts were studied. Our mRNA expression data indicate that TNF-α is more potent stimulator of MMP-1 and IL-1β of MMP-3 (Figure 9A), MMP-3 expression increasing 300-fold by it (Figure 9B). MMP-1 level has been shown to correlate with the degree of synovial inflammation (Maeda *et al.* 1995). In RA expression of TNF-α may precede that of IL-1β. This has been suggested by observations showing that IL-1β is particularly involved in advanced RA with progressive hard tissue degradation (Marinova *et al.* 1997, Abramson *et al.* 2002) and that in animal models of arthritis blocking of IL-1 reduces cartilage degradation and bone erosions, whereas blocking of TNF decreases synovitis (Strand and Kavanaugh, 2004). This has also been explained by uncoupling of inflammation from cartilage and bone tissue degradation in RA indicating that although inflammation would be under control, hard tissue destruction may proceed at least in part via mechanisms separate from those causing synovial inflammation (Van den Berg 1998). MMP-1-mediated degradation of cartilage type II collagen may be mediated, not by pannus, but by chondrocytes of the cartilage. Earlier studies of IL-1-driven animal models showed rapid and extensive cartilage damage and it has also been reported that IL-1 leads to proteoglycan loss and matrix degradation (van den Berg *et al.* 2002, Pettipher *et al.* 1986). On the contrary, TNF seemed to be a less potent inducer of matrix degrading enzymes than IL-1 and in TNF-driven disease cartilage damage may be influenced by IL-1 acting downstream from it. The conclusion is that TNF-α stimulated MMP-1 expression is involved in inflammation during early RA and IL-1β stimulated MMP-3 is involved in cartilage degradation in more advanced RA, when the cartilage degradation is driven by invasive pannus tissue. In a recent study TNF-mediated cartilage damage was indeed shown to be dependent on downstream located IL-1 and that IL-1 deficiency in mice decreased MMP-3 expression, suggesting that IL-1 and MMP-3 are the key factors in inflammatory cartilage tissue degradation (Zwerina *et al.* 2007).

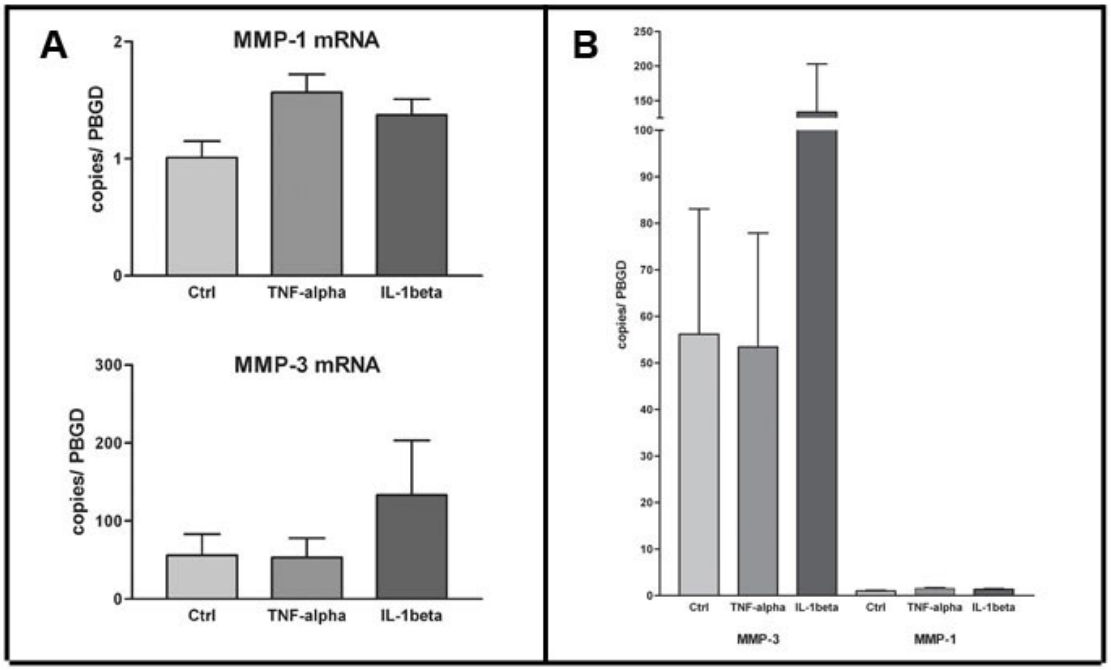

Figure 9. A) *Quantitative mRNA expression levels of MMP-1 and MMP-3, normalized for porphobilinogen deaminase in non-stimulated fibroblast cultures (Ctrl, n = 3) compared to TNF-α or IL-1β stimulated fibroblast cultures (n = 3). B) Quantitative mRNA expression levels of MMP-1 and MMP-3 normalized for porphobilinogen deaminase in non-stimulated (negative control, n = 3) and TNF-α and IL-1β stimulated (n = 3) fibroblast cell cultures shown in the same scale.*

IL-17 is one of the new potential cytokine targets in the management of RA. IL-17 expression has been shown to be increased in inflamed tissue (Beklen *et al.* 2007), but the expression levels were quite low, as also in our study in RA (Figure 10A). Expression levels of IL-17 were slightly increased in pannus tissue (Figure 10A), but IL-17 did not have any major influence on MMP-3 expression (Figure 10B). Increased IL-17 levels have been observed during the early stages of the disease in synovial fluid and synovial tissue (Kotake *et al.* 1999, Chabaud *et al.* 1999), but a recent study showed that less than 1% of T cells in RA synovium are positive for IL17A. It therefore seems that IL-17 is not a good stimulator candidate of fibroblast MMP-3 compared to TNF-α and IL-1β, but it might be involved in stimulation of TNF-α and IL-1β production (Beklen *et al.* 2007), particularly during the early stages of the disease and thus promote subsequent tissue destruction in RA. It has been suggested that IL-17 occupies a place at the top of the inflammatory cascade and stimulates other cells, such as

fibroblasts and macrophages, to increased production of proinflammatory cytokines. RA is not necessarily an IL-17-predominated disease, but it might be that IL-17 plays an important role in the early stages of RA (Raza *et al.* 2005). Because IL-17 and IL-17 receptor comprise several isoforms, this story may be unravelled in more depth in the future (Annunziato *et al.* 2009).

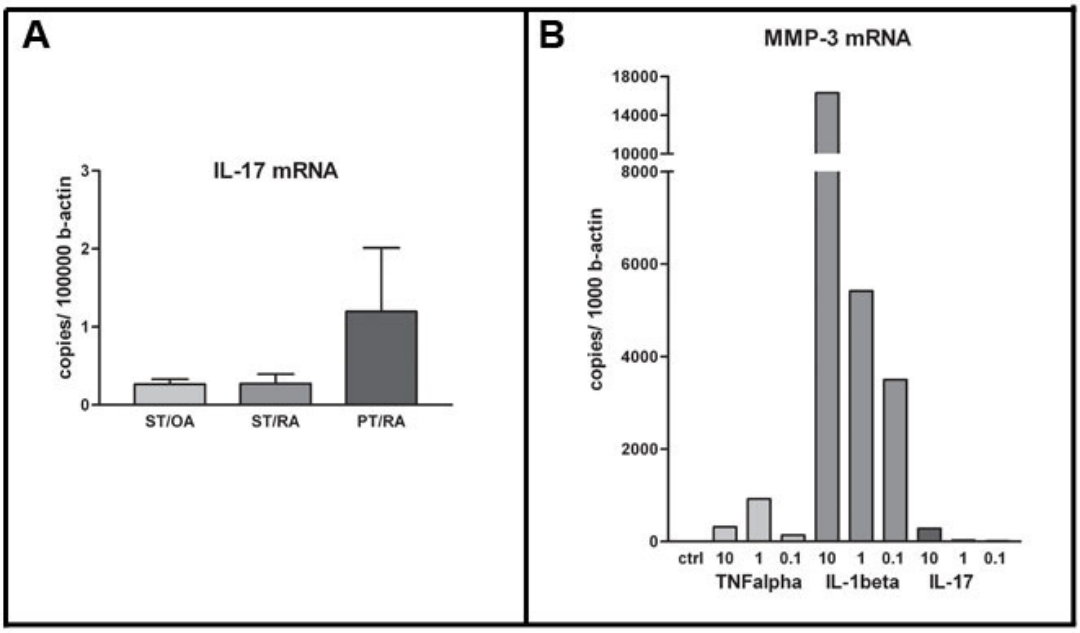

Figure 10. A) *Quantitative mRNA expression of IL-17 normalized for β-actin in osteoarthritic synovial tissue (ST/OA, n = 4), rheumatoid arthritic synovial tissue (ST/RA, n = 4) and pannus tissue (PT/RA, n = 4). B) Quantitative mRNA expression of IL-17 normalized for β-actin in nonstimulated (ctrl, n = 2) and 10, 1 and 0.1 ng/ml TNF-α, IL-1β or IL-17 stimulated (n = 2) fibroblast cell cultures.*

7.2 Cartilage degradation by pannus-derived proteinases (I, II)

In RA different proteinases can contribute to joint destruction by directly degrading the cartilage and to pannus invasion by degrading extracellular matrix and indirectly by activating other proteinases capable of doing so. Synovial fibroblast- and macrophage-like cells can release proteinases capable of digesting the cartilage matrix components and this destruction occurs primarily in areas close to inflamed pannus tissue in RA (Kobayashi and

Ziff 1975, Edwards 2000). The increased activity of proteolytic enzymes observed in arthritic joints may preceed the matrix degradation but may represent also a consequence of debris resulting from damaged cartilage and bone tissue.

It has been speculated that multiple members of the ADAMTS family may be important in connective tissue homeostasis and pathology. ADAMTSs cleave cartilage aggrecan mainly at the aggrecanase site (Glu373-Ala374), but some also secondarily at the matrix metalloproteinase site (Asn341-Phe342) (Westling *et al.* 2002). One of the aims of our study was to detect the eventual role of ADAMTSs in cartilage degradation. Because of earlier studies of involvement of ADAMTSs in aggrecan degradation and carcinomas, we studied the mRNA expression of ADAMTS-1, -4, -5, -12, -13 and -19 in RA and OA synovium as well as in pannus tissue. None of these ADAMTSs were increased in pannus tissue (Figure 11) and the expression levels were rather low compared to MMP levels (Figure 7B). Levels of ADAMTSs were normalized in these experiments for PBGD, because expression levels were almost undetectable when compared to β-actin. Later studies have shown an eventual involvement of ADAMTS-4 and -5 in arthritic diseases, but these ADAMTSs were isolated from cartilage (Nagase and Kashiwagi 2003) and have been implicated in aggrecan degradation (Malfait *et al.* 2002). Both ADAMTS-4 and -5 knockout mice were phenotypically normal and underwent normal development (Glasson *et al.* 2004, 2005; Stanton *et al.* 2005). In a model of inflammatory arthritis, only ADAMTS-5 knockout mice were protected against aggrecan loss, suggesting that ADAMTS-5 is responsible for the increased aggrecanase activity and cartilage pathology. However, *in vitro* studies using siRNA, neutralising antibodies and immunoprecipitation suggested a significant role also for ADAMTS-4 in aggrecan degradation (Fosang *et al.* 2008). The insignificant expression of ADAMTS in pannus tissue, but their increased expression in cartilage chondrocytes, implicates that they are derived from chondrocytes. These data suggest that rheumatoid cartilage destruction is partly mediated by pannus-derived MMP-3 and chondrocyte-derived ADAMTS-4 and -5. MMP-3 might precede ADAMTS-mediated degradation in early stages of RA, when pannus is starting to invade into cartilage and chondrocytes have not been activated yet.

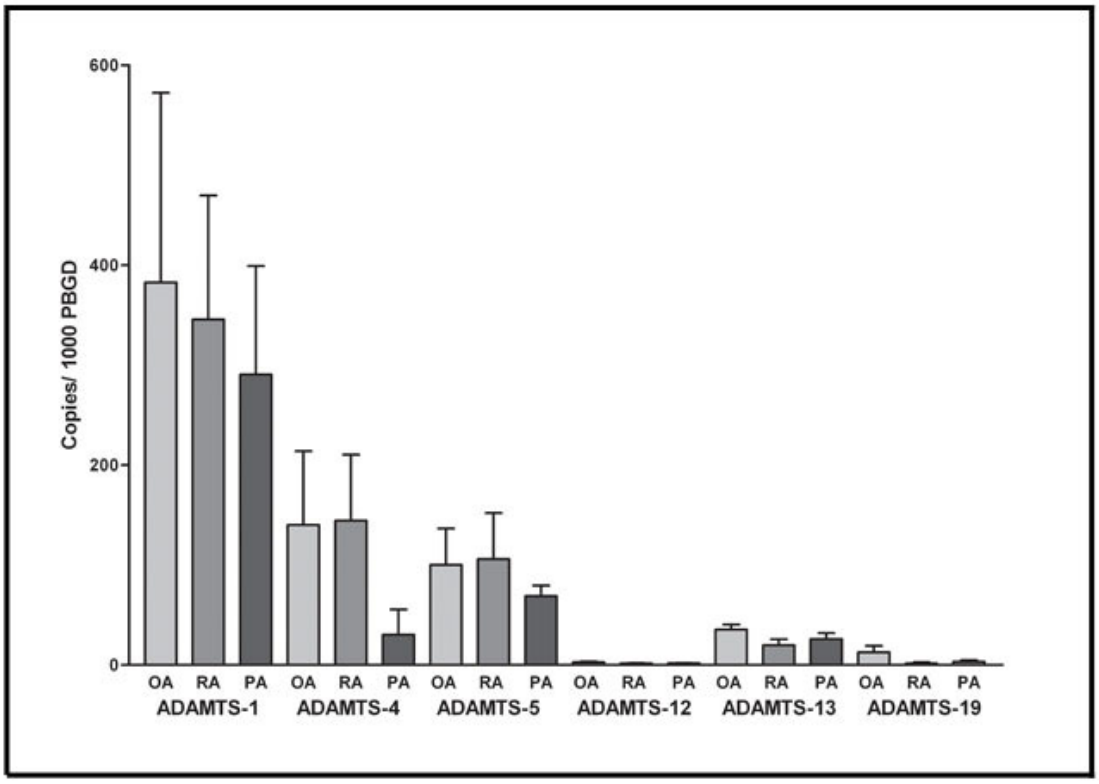

Figure 11. *Quantitative mRNA expressions of ADAMTS-1, -4, -5, -12, -13 and -19 normalized for PBGD in osteoarthritic synovial tissue (ST/OA, n = 4), rheumatoid arthritic synovial tissue (ST/RA, n = 4) and pannus tissue (PT/RA, n = 4).*

MMP-3 is not able to cleave collagen type I and II, but it has been shown to be important in cartilage proteoglycan (aggrecan) degradation. As was discussed earlier, MMP-3 is not involved in early stage RA, but more in advanced stages when degradation of cartilage occurs. The pH optimum of MMPs is usually between 7.5 and 8.0, but MMP-3 has a pH optimum between 5.5 and 6.0 so it can digest its substrates both in acidic and neutral environments. This lower pH optimum might be an advantage in pannus-mediated tissue degradation, because the junction between the pannus and hard tissue may develop a low pH. Measurements on cartilage affected by osteoarthritis demonstrate an acidic pH between 5.5 and 6.2 in this cartilage layer (Konttinen *et al.* 2002). Bone resorbing osteoclasts do not invade into non-mineralized cartilage but also these cells need an acidic pH for bone collagen degradation. Indeed, the acidic cysteine endoproteinase cathepsin K has been shown to be the key enzyme in osteoclastic bone resorption, but it may also play an important role in the

progressive destruction of articular cartilage, if the acid-base milieu enables activity (Konttinen *et al.* 2002).

The apparently low pH in cartilage-pannus junction is thus permissive, not only for MMP-3 activity, but also for cathepsin K and other cathepsins which are acidic endoproteinases. Inhibition of cathepsin L reduced cartilage destruction in a mouse arthritis model (Schedel *et al.* 2004) suggesting that these acidic enzymes are involved in the degradation of type II collagen. Cathepsins belong to the cysteine endoproteinase family. Their potential extracellular role is in the degradation of ECM proteins, such as fibronectin, laminin and collagen types I, II, IV, XVII, and bone matrix proteins, such as osteopontin, osteocalcin and osteonectin as well as in the activation of proenzymes. Based on the capability of cathepsins to degrade collagen, we investigated the mRNA expression of cathepsins K, L and B in pannus tissue compared to RA and OA synovium. All these cathepsins have been previously found in synovium and at the soft-hard tissue junction in RA (Trabandt *et al.* 1990 and 1991, Hummel *et al.* 1998, Kaneko *et al.* 2001), but our results do not suggest that they play a role in pannus, because their expressions levels were highest in RA synovium and second highest in OA synovium being relatively low in pannus tissue (Figure 12A). Similarly, the expression levels were higher in primary cultures of RA synovial fibroblasts than in primary cultures of pannus fibroblasts. The most potent stimulator of cathepsin L and B in RA synovial fibroblasts in 48-hour experiments was IL-17, which had low expression in pannus tissue as shown previously, but which in *in vitro* experiments was even more potent stimulant than TNF-α and IL-1β (Figure 12B). Interestingly, cathepsin B was the predominant cathepsin in monocyte/macrophages, while cathepsin L was 10 times lower and cathepsin K was not detected at all. Previous data also shows cathepsin L and B expression in monocytes (Reddy *et al.* 1995) and that cathepsin B is capable to activate pro-MMP-3 (Murphy *et al.* 1992). Inhibition of cathepsin L protein synthesis in a mouse arthritis model reduced cartilage destruction (Schedel *et al.* 2004). However, all these cathepsins are also expressed in chondrocytes suggesting that they might play a role in chondrocyte-mediated cartilage remodelling and destruction (Lang *et al.* 2000, Konttinen *et al.* 2002). In contrast, it seems that cartilage degradation is not mediated by cathepsin derived from pannus itself. Cathepsins K, B and L have been shown to degrade type II collagen and proteoglycans so they can contribute to cartilage degradation (Maciewicz *et al.* 1990, Maciewicz and Wotton 1991, Hou *et al.* 2001). Another explanation for the role of cathepsins found in synovial fibroblasts could be their involvement in the degradation of endocytosed cartilage collagen (Hou *et al.*

2001) after other neutral endoproteinases, like MMPs, have first partly degraded cartilage collagen fibre matrix, followed by endocytosis by fibroblasts (or macrophages).

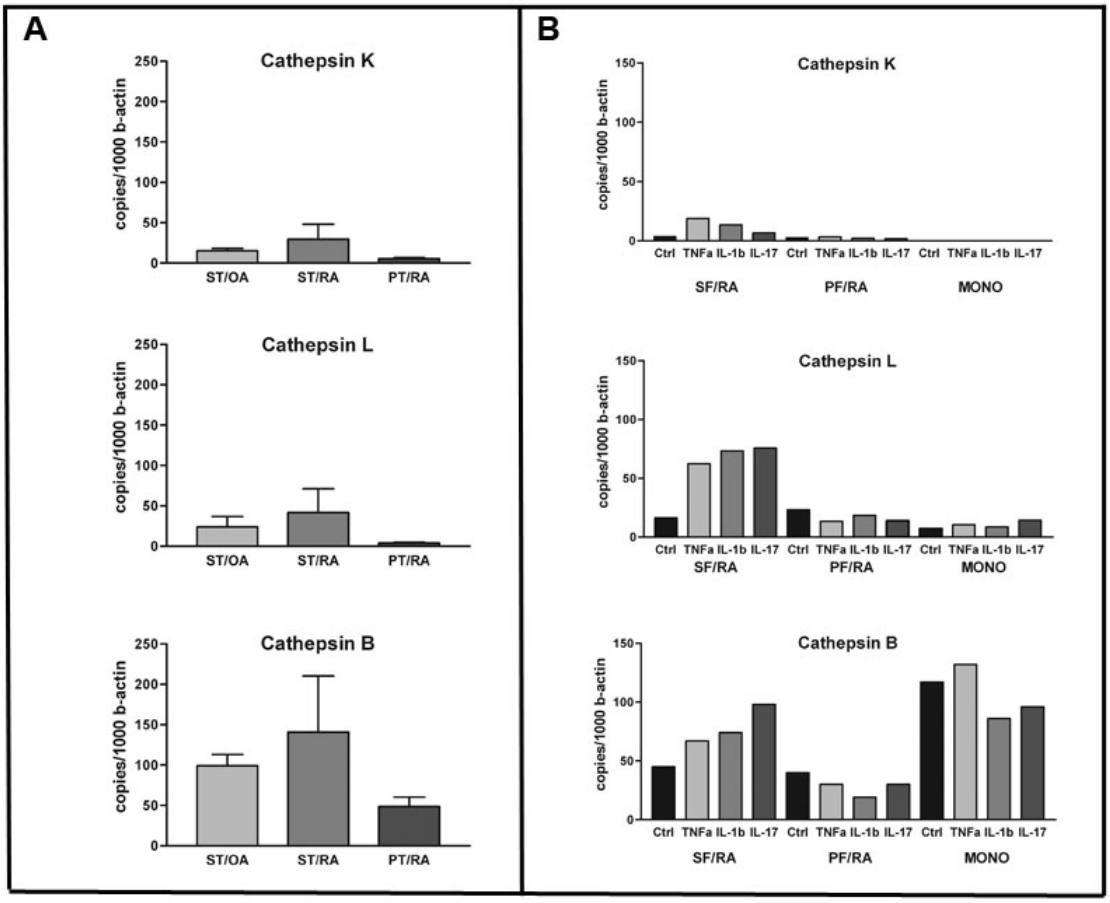

Figure 12. A) *Quantitative mRNA expressions of cathepsin K, L and B normalized for β-actin in osteoarthritic synovial tissue (ST/OA, n = 9), rheumatoid arthritic synovial tissue (ST/RA, n = 9) and pannus tissue (PT/RA, n = 8). B) Quantitative mRNA expression of cathepsin K, L and B normalized for β-actin in nonstimulated (ctrl, n = 2) and TNF-α, IL-1β or IL-17 stimulated (n = 2) rheumatoid arthritic synovial fibroblasts (SF/RA), pannus fibroblasts (PF/RA) and monocytes from peripheral blood (MONO) at 48 hours.*

7.3 Osteoclast formation (IV)

Multinuclear bone resorbing osteoclasts are formed by fusion of mononuclear progenitor cells. Before the fusion process cells are involved in a series of steps to achieve fusion competence. First some signal initiates differentiation leading to changes in cell phenotype

and shape. Before the final fusion steps the cells also have to recognize their fusion partner for proper adherence.

The differentiation of mononuclear progenitors to fusion competence is stimulated by M-CSF via c-fms receptor and by RANKL via its receptor RANK expressed on these prefusion cells. In our studies both human peripheral blood mononuclear cells and mouse monocyte-macrophage RAW 264.7 cells were used as the source of progenitor cells. We also explored if RANKL and its decoy receptor OPG are expressed in pannus tissue. Immunological staining of RANKL indicated that it is present in pannus tissue, often located close to multinuclear cells expressing TRAP used as an osteoclast marker. RANKL staining and the presence of multinucleated cells in areas where pannus invades into bone in RA has been indicated in other studies (Gravallese *et al.* 2000). We also detected increased RANKL mRNA expression in pannus tissue compared to RA and OA synovium, which is in line with the increased expression of corresponding protein in pannus. Messenger RNA expression of OPG was also detected, which would tilt the balance to the opposite direction. When RANKL was increased, its inhibitor factor OPG was decreased, which we conclude promotes osteoclastogenesis (Figure 13A). This has been confirmed also by other groups (Fonseca *et al.* 2005, Pettit *et al.* 2006). In normal knee joints, excessive expression of OPG over RANKL has been measured, suggesting the role of OPG in protection against joint damage (Smith *et al.* 2003). However, much of OPG may be localized in the vascular endothelial cells (Mandelin *et al.* 2003b) and may there participate in the interactions between endothelial RANK and vascular smooth muscle cells and mast RANKL rather than osteoclastogenesis (Ali *et al.* 2006). Next we wanted to study RANKL expression in pannus fibroblasts and to study the effect of different stimulators on RANKL expression. RANKL expression levels were higher in pannus fibroblasts than in RA synovial fibroblast (Figure 13B) and all the stimulants used, TNF-α, IL-1α and IL-17, increased RANKL expression in fibroblasts (Figure 13C), TNF-α being the most effective stimulant, which has been recently also observed by another group (Tunyogi-Csapo *et al.* 2008).

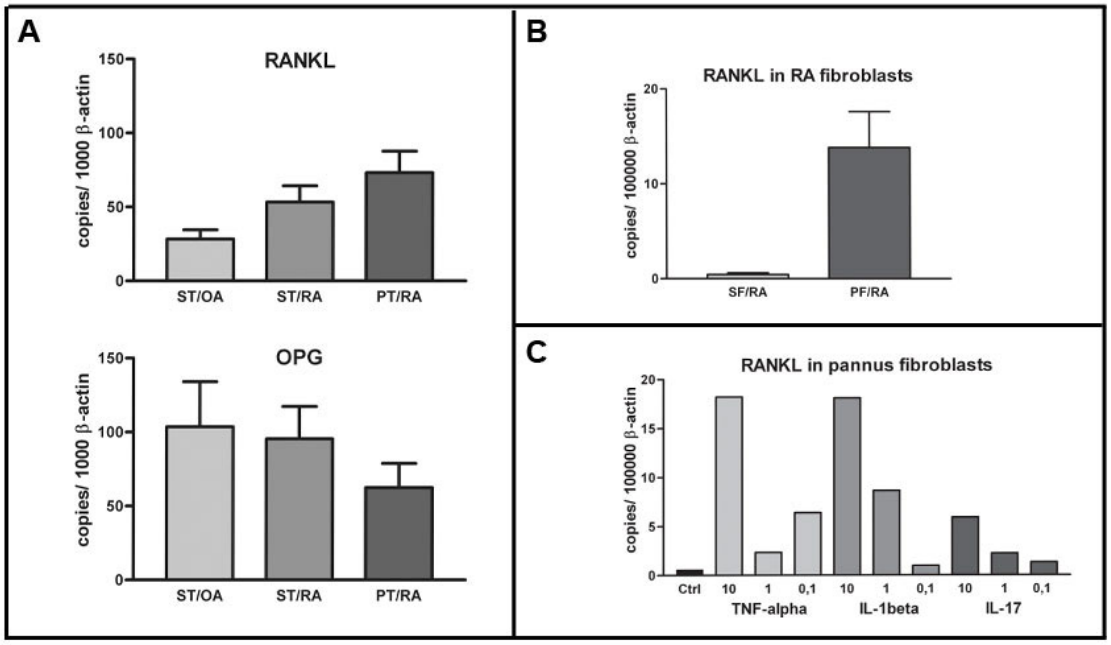

Figure 13. *A) Quantitative mRNA expressions of RANKL and OPG, normalized for β-actin in osteoarthritic synovial tissue (ST/OA, n = 9), rheumatoid arthritic synovial tissue (ST/RA, n = 9) and pannus tissue (PT/RA, n = 8). B) Quantitative mRNA expressions of RANKL normalized for β-actin in rheumatoid arthritic synovial fibroblasts (SF/RA, n = 3) and pannus fibroblasts (PF/RA, n = 3). C) Quantitative mRNA expressions of RANKL, normalized for β-actin in nonstimulated fibroblasts (ctrl, n = 2) and fibroblasts stimulated with 10, 1 and 0.1 ng/ml TNF-α, IL-1β and IL-17 (n = 2).*

It is known that the essential signalling pathway for osteoclastogenesis involves NF-kappa B. RANKL binding to RANK on the surface of osteoclast precursors leads to NF-kappa B activation, via tumor necrosis factor receptor-associated factors (TRAFs) and translocation to the nucleus to activate the transcription factors for osteoclastogenic gene expression. TNF-α can also induce NF-kappa B signalling by direct binding to its own receptor (Li *et al.* 2008). Stimulation of cells with TNF-α triggers activation of NF-kappa B through various signalling molecules, which include TRAF. The negative regulation of NF-kappa B signalling is essential for the normal function of cells and failure to down-regulate the NF-kappa B-mediated signalling may lead to chronic inflammation and this might be the main effect of increased TNF-α levels in RA, as earlier stated. Osteoclastogenesis can be triggered by IL-17 through the up-regulation of RANKL on fibroblasts and other cells through IL-17 receptor induced NF-kappa B activation (Sato *et al.* 2006, Kotake *et al.* 1999). Deficiency of IL-17

alone did not reduce inflammatory bone destruction (Tunyogi-Csapo *et al.* 2008), but IL-17 is not only capable to induce RANKL production, but it can also induce proteinases and can that way contribute to cartilage and bone degradation.

To confirm fibroblast function in osteoclast formation, we co-cultured osteoclast precursor cells with pannus fibroblasts with M-CSF but without RANKL to follow the eventual formation of multinuclear $TRAP^+$ osteoclast-like cell. This interaction might be the result of cell-cell contact mediated via RANKL/RANK interaction or via soluble RANKL produced by fibroblasts. It has been earlier reported that RA synovial fibroblasts express higher than normal levels of RANKL and seem therefore to induce osteoclast-like cells more effectively than synovial cells expressing only low levels of RANKL (Shigeyama *et al.* 2000). We also checked if TNF-α, IL-1β and IL-17 stimulated monocyte/macrophages express RANKL, but none was observed although it has been shown that numerous cell types in tissue of collagen-induced arthritis in mice, including macrophages and fibroblasts, are capable to produce RANKL (van den Berg *et al.* 2007). This difference might be due to distinct activation routes in cytokine stimulated cultured cells and in collagen induced arthritis. Inhibition of RANKL by its natural inhibitor OPG in mouse models confirms the role of this factor in osteoclastogenesis by preventing peri-implant osteolysis (Goater *et al.* 2002) and formation of erosions in RA (Kong *et al.* 1999).

After initial differentiation the mononuclear precursor cells should come into direct cell-cell contact with other fusion competent cells. It has been suggested that some members of the ADAM family contain a putative fusion peptide responsible for promotion of cell fusion upon cell-cell contact. Because it had already been reported that ADAM8, 9 and 12 seem to be involved in the formation of multinuclear osteoclasts (Yagami-Hiromasa *et al.* 1995, Namba *et al.* 2001, Choi *et al.* 2001), we wanted to investigate first the mRNA expression of these ADAMs in the bone-pannus junction. Only ADAM8 showed a clearly increased expression in pannus tissue compared to RA and OA synovial tissues and ADAM9 was even more highly expressed in OA synovial samples (Figure 14A). As mentioned, it had been earlier suggested that ADAM8 is involved in osteoclast formation and that its inhibition in mouse bone marrow culture decreases osteoclast formation (Choi *et al.* 2001) and its role in cell fusion is also supported by a recently published study from our group (Ma *et al.* 2009). Therefore, we focused our studies to ADAM8 and next observed that, apart of its already

mentioned increased mRNA expression (Figure 14B), also the corresponding protein was increased (Figure 14C) in pannus tissue invading into bone. During the formation of

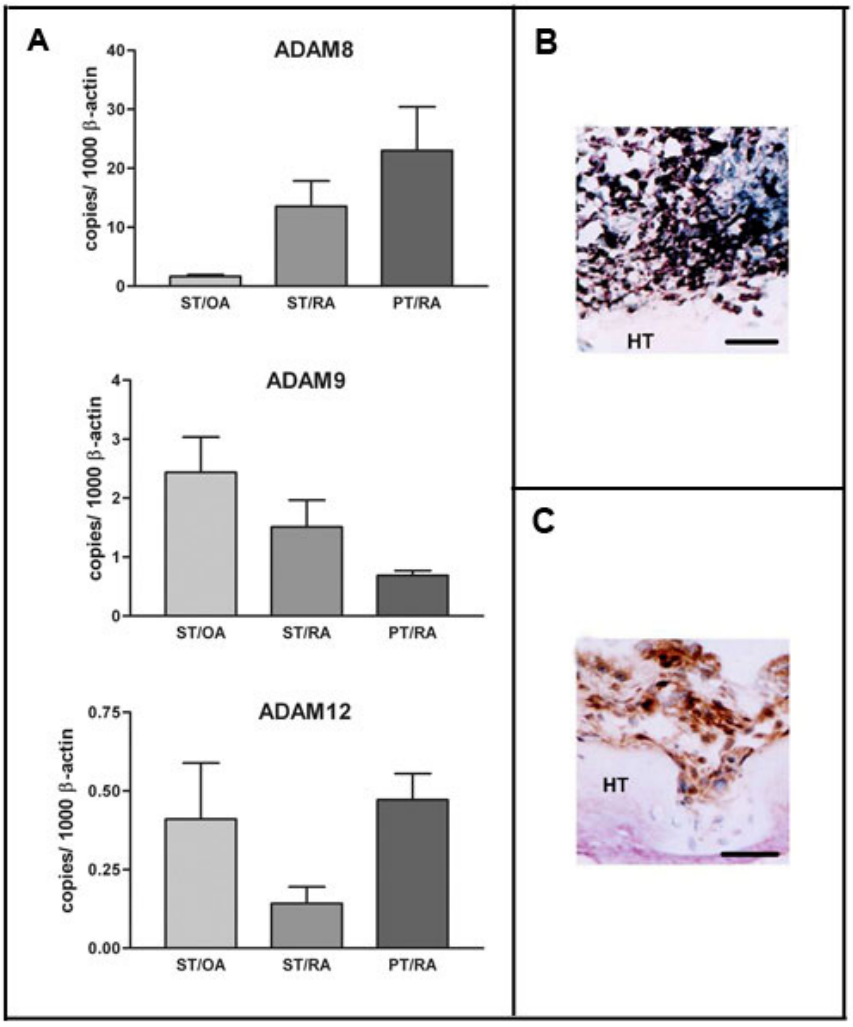

Figure 14. *A) Quantitative mRNA expressions of ADAM8, -9 and -12 normalized for β-actin in osteoarthritic synovial tissue (ST/OA), rheumatoid arthritic synovial tissue (ST/RA) and pannus tissue (PT/RA). B) In situ hybridization of ADAM8 mRNA at pannus-hard tissue junction. C) Immunohistochemical staining of ADAM8 protein at the cartilage/bone-pannus junction. Scale bar 50 µm. HT, hard tissue.*

multinuclear osteoclasts, ADAM8 expression was increased (Figure 15A and B), however in multinuclear cells, the expression started to diminish (Figure 15A, days 7 and 9), indicating that it is functioning in forming osteoclasts. ADAM8 function was studied in mouse macrophage RAW cells by silencing ADAM8 expression with small interfering RNA

(siRNA) duplex oligos and by increasing its expression using transfection with a cloned full-length ADAM8 vector. The eventual effect of both procedures on osteoclast formation was assessed by measuring mRNA expression of several osteoclast markers, namely TRAP, calcitonin receptor, integrin β3 and cathepsin K. These markers were decreased and increased during stimulated osteoclastogenesis upon silencing and transfection of ADAM8, respectively, and also TRAP staining indicated decreased fusion of the ADAM8 silenced

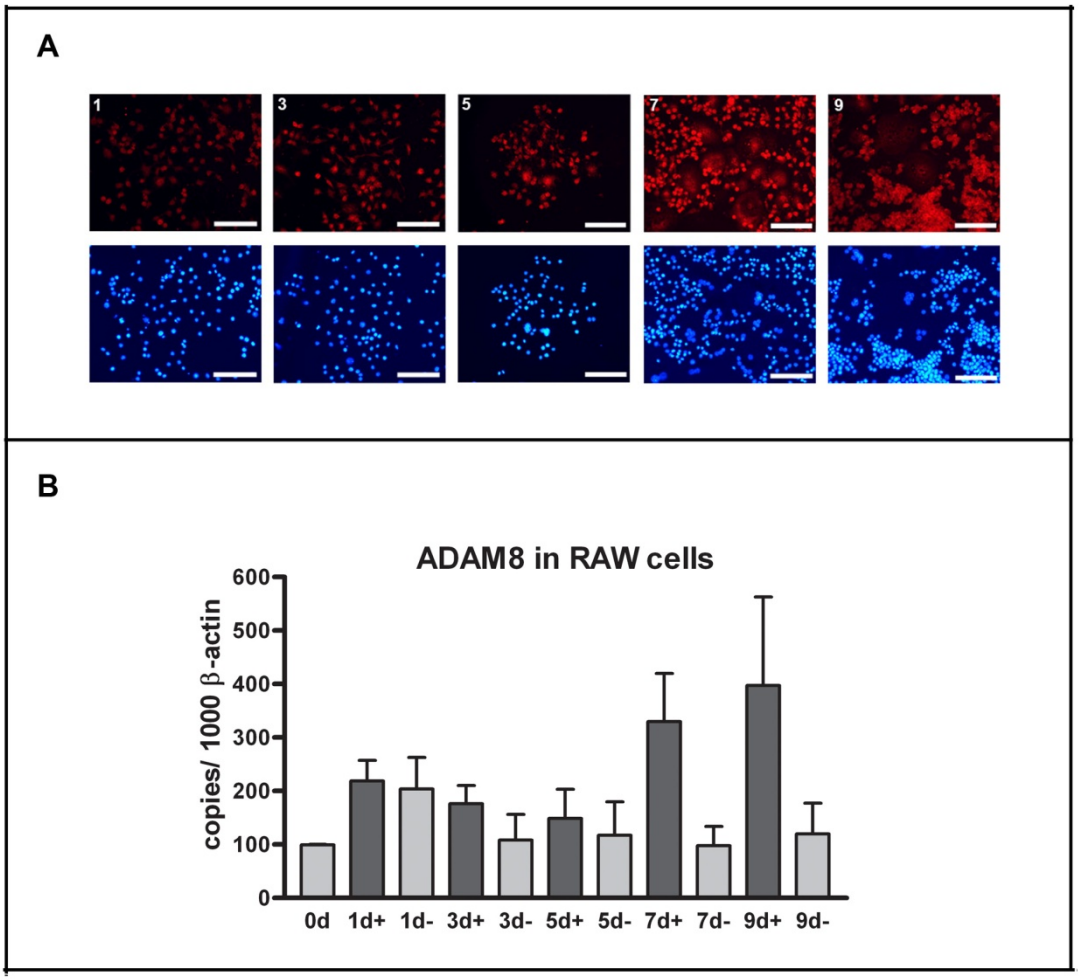

Figure 15. A) *Immunohistochemical staining of ADAM8 (red colour) protein on RANKL stimulated RAW 264.7 macrophage cell cultures during their differentiation into osteoclast-like multinuclear cells at 1, 3, 5, 7 and 9 days. Nuclei are visualized by DAPI staining (blue colour). Scale bar 100 μm. B) Quantitative mRNA expression of ADAM8 normalized for β-actin in non-treated controls (-) and RANKL stimulated (+) RAW 264.7 macrophage cell cultures during their differentiation into osteoclast-like multinuclear cells. The duration of stimulation is indicated in days (n = 3).*

cells. The effects of cytokines on ADAM8 mRNA and protein expression during osteoclastogenesis, detected using quantitative PCR and flow cytometry, pointed out that at least IL-1β and M-CSF play a role for ADAM8 expression. The modular ADAM8 protein is a transmembrane protein, which contains a pro-domain followed by a metalloproteinase domain and is further in the C-terminal direction flanked by potential adhesion and fusion active disintegrin and cysteine-rich domains. These are further followed by a transmemrane domain and an intracellular domain. Catalytic metalloproteinase activity of ADAM8 resembles that of the MMPs, such as MMP-2, MMP-3 and MMP-13 (Yamamoto *et al.* 1999, Amour *et al.* 2002) and the substrate specificity at least for proteoglycans occurs also in low pH optimum, which might favour the tissue degradation in junction between the acidic pannus and hard tissue (Hall *et al.* 2009b). For the release of the active site of the metalloproteinase domain the blocking pro-peptide domain has first to be cleaved off and, similarly, for the release of the fusion active domain, the metalloproteinase domain should be removed (Figure 16A). We studied this aspect with Western blotting using an antibody against the intracellular domain of the ADAM8 protein. Indeed, we noticed that before (and during) the formation of multinuclear osteoclasts the pro-domain was first cleaved off and this was followed by removal of the metalloproteinase domain as if to release the activity of the putative fusion active domain (Figure 16B). Based on studies utilizing deleted forms of ADAM8 it has been concluded that the direct cell-to-cell contact is mediated via its disintegrin/cysteine-rich domains (Choi *et al.* 2001). Not only the cysteine-rich domain, but also the epidermal growth factor-like domain, found in some other ADAM family members, are both homologous to viral fusion proteins and are involved in membrane fusion (Huovila *et al.* 1996). Interactions between the disintegrin domain of ADAM8 and integrin receptors on osteoclast precursor cells may be the event triggering cell membrane and cellular fusion. The disintegrin domain of ADAM8 does not contain RGD sequences, but it can interact with alpha9-beta1 integrin, which is expressed on osteoclast precursors and which increases during their differentiation to osteoclasts (Rao *et al.* 2006). However, it has been reported that the soluble form of ADAM8 can also induce cell fusion and it can therefore be concluded that the cell-to-cell contact is not the only way ADAM8 may potentially support formation of multinuclear cells. Increased expression of ADAM8 has also been found in synovial fluid of RA patient and its concentrations correlate with the degree of joint inflammation (Gomez-Gaviro *et al.* 2007). These data make ADAM8 a potential therapeutic candidate molecule, which could first mediate cartilage matrix destruction due to its proteolytic properties and

subsequently increase bone degradation due to its potential role in osteoclastogenesis during development of bone erosions in RA.

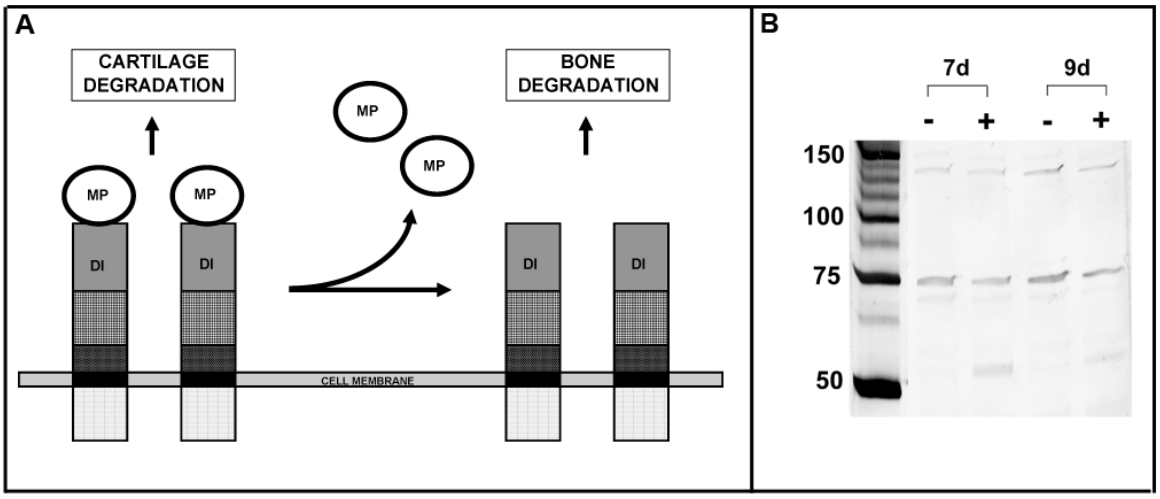

Figure 16. *A) On the left the proteolytically active ADAM8 mediates degradation of cartilage after cleavage of the pro-domain exposing the metalloproteinase domain (MP). Upon further removal of the metalloproteinase domain the fusion active disintegrin domain (DI) able to mediate osteoclastogenesis is released, which leads to the formation of osteoclasts and osteolysis. B) Conversion of ADAM8 pro-form (120 kD) into proteolytically active metalloproteinase (75 kD) and further to fusion-active disintegrin (55 kD) in RANKL stimulated (+) RAW 264.7 macrophage cultures at 7 and 9 days assessed using Western blots compared to non-stimulated (-) RAW cells.*

7.4 Bone degradation (V)

Our previous data showed only minor expression of cathepsin K in pannus tissue, which might indicate that only a few osteoclasts are involved in the formation of erosions, but which also made it necessary to study this in more detail. To do this a pycnodysostosis patient with bone erosions was analyzed. Her peripheral blood monocytes were studied for their ability to mediate cathepsin K-independent bone resorption.

Osteoclasts are responsible for bone resorption and bone degradation is thus regulated by their number and activity level. Multinuclear cells, with osteoclastic phenotype expressing calcitonin receptor, cathepsin K and TRAP, are found in pannus tissue adjacent to bone

(Bromley and Woolley 1984, Gravallese *et al.* 1998, Goldring and Gravallese 2000). Synovial fibroblasts and T-cells are considered to be the primary source of osteoclastic factors in synovial membrane. Mature osteoclasts, together with activated macrophages, mediate bone destruction by secreting proteinases, including MMPs and cathepsins, which degrade demineralised bone matrix. Cathepsin K is known to be a one of the most important proteinases involved in osteoclast-mediated bone loss and cathepsin K has been used as an osteoclastic marker to verify formation of multinuclear osteoclastic cells. Cathepsin K mRNA expression increases during osteoclastogenesis. In RA cathepsin K-positive cells have been observed in hypertrophic synovitis tissue and at sites of cartilage and bone degradation (Hummel *et al.* 1998, Gravallese *et al.* 2000, Hou *et al.* 2001). Cathepsin K is also found in synovial fibroblasts and its expression is increased in RA synovitis tissue compared to control synovial tissue (Hou *et al.* 2001). Serum cathepsin K levels correlate with radiological progression of bone destruction (Skoumal *et al.* 2005). Cathepsin K has also been reported to be present in macrophages in RA (Hou *et al.* 2001). Noteworthy, our experiments showed no cathepsin K mRNA expression in unstimulated healthy monocyte/macrophages or TNF-α, IL-1β and IL-17 stimulated monocyte/macrophages at 48 hours. In our study we analyzed cathepsin K mRNA expression and it was not increased in pannus tissue or in pannus-derived fibroblasts compared to RA and OA synovial tissue (Figure 12A) or synovial fibroblasts derived from such tissues (Figure 12B). Synovial fibroblasts can internalize collagen type II and accumulate it within their lysosomes, suggesting the cathepsin K postitive fibroblasts might play a role in the clearance of endocytosed collagen at the site of cartilage erosion (Hou *et al.* 2001) and a similar process could happen in the bone-pannus junction also for collagen type I.

In human osteoclasts, that express little or no cathepsin B and L, it is supposed that cathepsin K mediates degradation of collagen type I and other bone-matrix-associated proteins like osteopontin, osteocalcin and osteonectin. If cathepsin K production or activity is inhibited, osteoclasts most probably are not able to mediate bone collagen matrix degradation. This is why the involvement of cathepsin K in bone degradation was studied more closely by using peripheral blood mononuclear cells from our pycnodysostosis patient. Pycnodysostosis is characterized by cathepsin K deficiency and due to the lack of cathepsin K osteoclasts of pycnodysostosis patients can not maintain normal bone remodelling leading to impaired development of the skeleton (short stature), ineffective bone resorption and abnormally dense bones (osteosclerosis). However, in spite of this it became evident that our pycnodysostosis

patient, who by accident had also developed an inflammatory arthritis, developed bone erosions (Figure 17). This "Experiment of Nature" represents a "human cathepsin K knock-out" arthritis model. Interestingly, a recent study indicates that cathepsin K knock out does not protect mice against inflammatory bone erosions in human TNF-transgenic mouse model

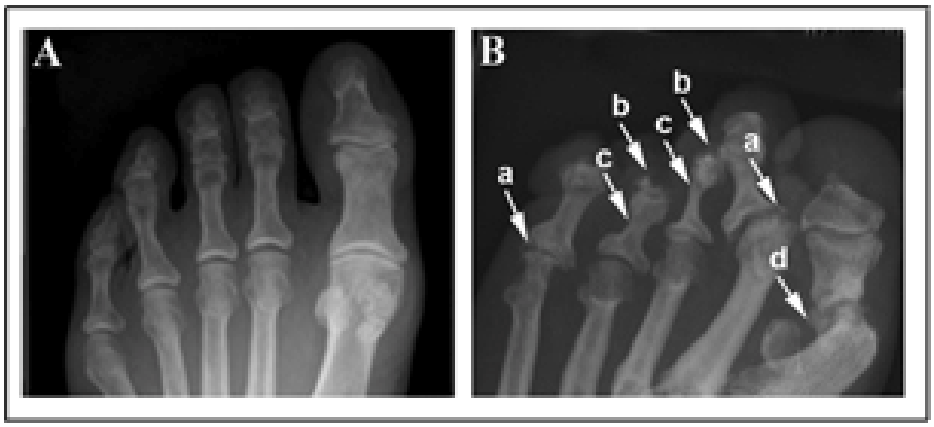

Figure 17. *Bone erosions and acro-osteolysis in toes of the pycnodysostosis patient suffering from psoriatic arthritis. X-ray of normal toes (A) and of a pycnodysostosis patient from year 2005 (B). Panel B demonstrates patient's foot with erosive changes in II and V MTP joints (a), complete resorption of the distal phalanges of digits III-IV (b) and partial resorption of the mid phalanges of digits III and IV (c). Also malunion after an attempted arthrodesis of the I MTP joint can be seen.*

(Schurigt *et al.* 2008). Serum of our pycnodysostotic arthritis patient was analysed for collagen degradation products generated by cathepsin K (CTx for cross-linked collagen telopeptides produced from mature collagen fibers by the action of cathepsin K) and for collagen degradation products generated from mature collagen fibers by collagenolytic MMPs (ICTP for type I collagen telopeptides). The results showed only very low CTx values, within the limits of the method background values, but high ICTP values further verifying cathepsin K deficiency. Mononuclear cells were differentiated into multinuclear osteoclasts, which were analyzed for cathepsin K, TRAP and calcitonin receptor as osteoclast markers. These results indicate that TRAP and calcitonin receptor expression were similar to those of the healthy controls, but as supposed, cathepsin K was missing in patient samples. Formation of multinuclear TRAP positive osteoclast-like cells was similar in our pycnodysostosis patient and our healthy control on both culture plates and on dentine discs (Figure 18A and

B). Thus, lack of cathepsin K did not seem to much affect the formation or the number of multinuclear cells. However, the analysis of the capability of the osteoclast-like cells to erode bone discs showed that the resorption pits formed different patterns. Osteoclasts from the healthy control formed normal resorption pits with trails (Figure 18D), but the osteoclasts from our pycnodysostosis patient formed resorption pits without trails as if they had lost their ability to migrate on the surface of the dentine slice (Figure 18E). This must be somehow connected to the loss of cathepsin K. Therefore, it was next tested how inhibition of cathepsin K activity in healthy control osteoclasts affects formation of resorption pits. The results of these experiments further suggest that cathepsin K participates in osteoclast migration, because formation of multinuclear cells was not affected (Figure 18C). Furthermore cathepsin K-inhibited osteoclasts showed the same pattern as osteoclasts generated from the osteoclast precursors of the pycnodysostosis patient, being apparently rather sessile on dentine slices (Figure 18F). As almost all complex cellular phenomena are dependent on interplay between

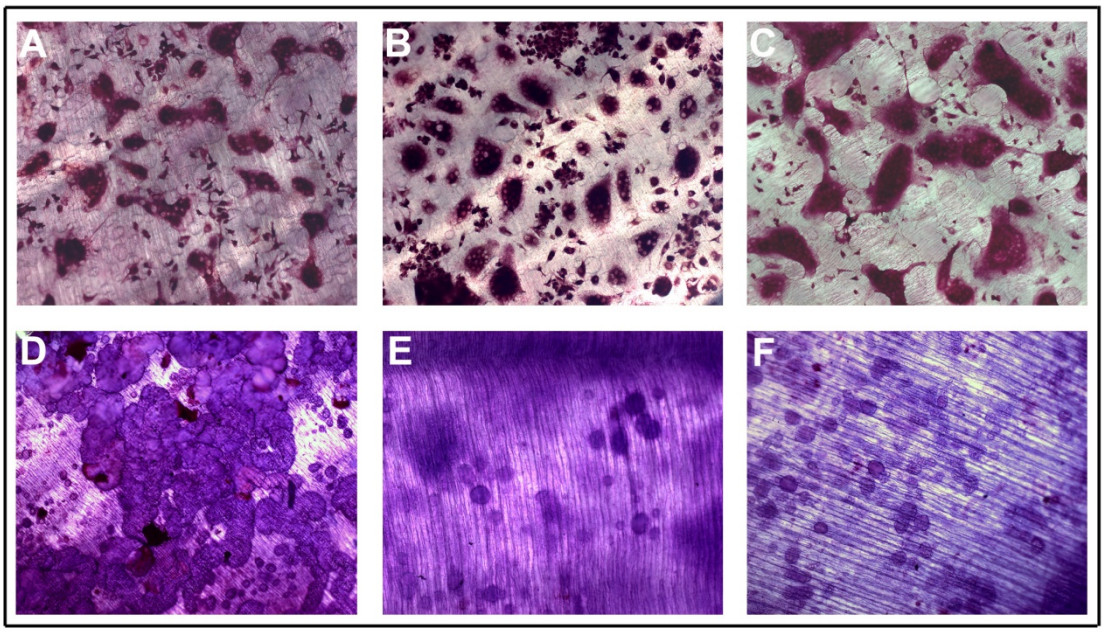

Figure 18. *Bone resorption by M-CSF and RANKL induced human osteoclasts. Immunohistochemical staining of the osteoclast marker TRAP in multinuclear cells from (A) a normal control, (B) pynodysostosis patient and (C) in cathepsin inhibited multinuclear cells from a normal control cultured on dentine discs. Toluidine blue staining of resorption pits formed by the corresponding (D) normal control cells, (E) pynodysostosis cells and (F) cathepsin inhibited normal control cells on dentine discs.*

several molecules, it is concluded that cathepsin K, although necessary for osteoclast migration, is probably not the only relevant factor in it.

Cathepsin K activity and collagen degradation products CTx and ICTP were measured over time in cell culture supernatants from dentine disc, which contained osteoclasts produced from cells of the healthy control or the pycnodysostosis patient. As supposed, active cathepsin K levels of the pycnodysostosis osteoclast cultures were under detection level. This was accompanied by lack of CTx type collagen degradation products, whereas the ICTP levels in the culture supernatants were higher than in the control samples. This might be due to a compensatory and increased MMP-mediated degradation of bone and/or missing of the second cathepsin K-mediated degradation step of ICTP to CTx, which however both represent a consequence of the cathepsin K deficiency. In line with this reasoning, a recent study suggests that the loss of cathepsin K can be compensated in addition to other MMPs and cysteine proteinases (Everts *et al.* 2006), also by some still unknown proteinases. This study may also suggest that different sites of bone contain different osteoclasts, which could use different sets of proteinases for bone matrix degradation. Chondroclasts could belong to one of these types, which are shown to degrade mineralized cartilage and which resamble osteoclastic cells (Schenk *et al.* 1967). Osteoclast is the primary bone resorbing cell, but on the other hand, synovium-derived proteases may participate directly in the degradation of cartilage and bone in RA (Jones *et al.* 2008). Also fibroblast-like cells may be involved in bone resorption (Pap *et al.* 2003). Fibroblasts were shown to release acidic molecules to lower the pH in their immediate surrounding, which could lead to acidification of the microenvironment between the pannus and cartilage/bone, demineralization of the calcified bone matrix and activation of the acidic MMP-3 and cathepsin K during pannus invasion. Breast cancer cells can participate in degradation of organic bone matrix (Parikka *et al.* 2005), which also support the involvement of invasive synovial fibroblast-like cells in cartilage/bone degradation. Based on these results, it is concluded that although cathepsin K plays a pivotal role in the progressive destruction of bone matrix, it is not solely responsible for osteoclastic bone resorption, but also other proteinases are involved.

8. SUMMARY AND CONCLUSIONS

Summary I: Matrix metalloproteinases are involved in extracellular matrix degradation and analysis of 16 different MMPs in RA synovium revealed the presence in particular and eventual role of MMP-1, MMP-3, MMP-13 and MMP-14 in the development of RA when compared to the synovial tissue samples obtained from trauma patients.

Summary II: More detailed analysis of MMP-1, MMP-3, MMP-13 and MMP-14 indicated that among these MMPs the most likely enzymes involved in pannus tissue mediated matrix degradation are MMP-1 and MMP-3. Both were increased in pannus, but MMP-3 was the predominant one. Stimulation studies suggest TNF-α as a potent stimulator for MMP-1 and IL-1β for MMP-3. Based on the present results and some recent findings, it is concluded that MMP-1 is involved in inflammation and pannus tissue expansion during early RA, while involvement of MMP-3 in cartilage degradation becomes more prominent in advanced RA. In bone degradation also the eventual role of osteoblast produced MMPs, like MMP-13 and MMP-14, should be taken under consideration in RA. It can also be concluded that various stimulators and effectors are involved in the progression of RA during different stages. The eventual presence and participation of ADAMTSs in cartilage degradation was also studied, but these analyses suggest that they do not have any major involvement in pannus-mediated cartilage remodelling and degradation.

Summary III: Osteoclastic differentiation factor RANKL was characterized in pannus tissue and its expression was found to be increased in pannus compared to synovial tissues of RA and OA, while the expression of its natural inhibitor osteoprotegerin (OPG) was diminished in pannus tissue, indicating that the increased RANKL/OPG ratio may drive formation of multinuclear osteoclasts in pannus tissue. Indeed, immunopathological studies disclosed multinuclear osteoclast-like cells in the advancing edge of the pannus tissue close to bone matrix and in close contact to RANKL positive cells. These data indicate that during advancing pannus tissue invasion into bone matrix, the bone degrading osteoclasts are locally formed in pannus.

Summary IV: This study suggests a role for ADAM8 in cell fusion during the formation of multinuclear osteoclast-like cells. ADAM8 expression was increased locally in pannus tissue.

These observations support the conclusion of the previous study suggesting local osteoclastogenesis in pannus tissue and their role in the formation of rheumatoid erosions.

Summary V: Cathepsin K has been shown to be a key enzyme in normal osteoclastic bone resorption and it has probably an important role in the progressive destruction of articular cartilage in RA. However, it was found that the invading soft pannus tissue contains less cathepsin K than control synovial tissue although cathepsin K may be specifically located in and under pannus osteoclasts. It was shown that a pycnodysostosis patient, who also had arthritis, developed bone erosions in spite of her cathepsin K deficiency. These observations led to the hypothesis that apart from cathepsin K also other proteinases are involved in rheumatoid hard tissue degradation.

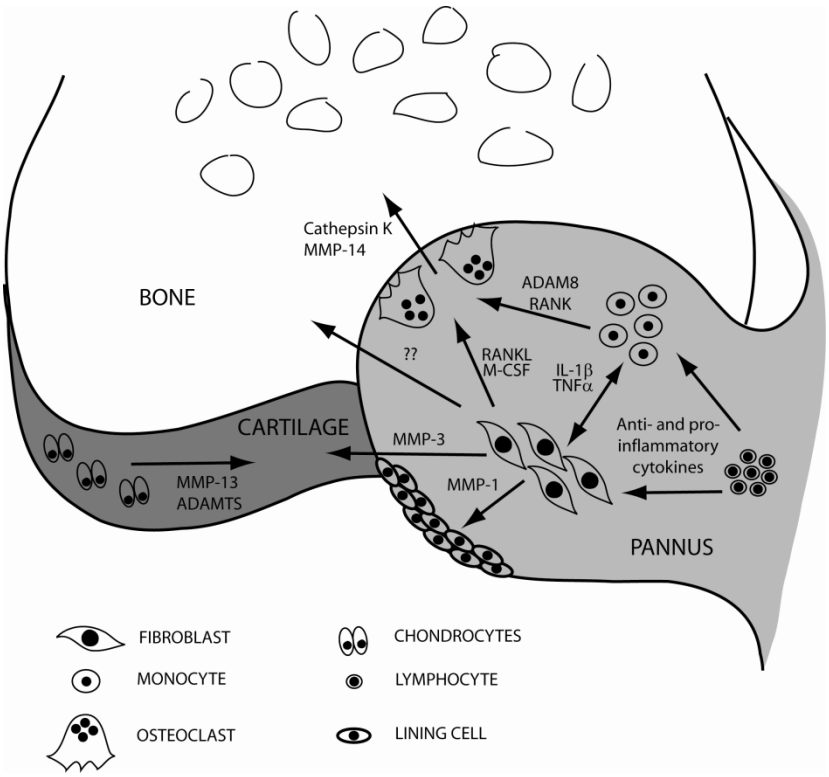

Figure 19. *Schematic picture summarizing the proposed roles of factors presented in this work in pannus derived cartilage and bone degradation in RA.*

Several proteinases have been found and each of them most probably has a specific role in normal homeostasis and in various destructive diseases. On the other hand, there is also redundancy and overlap between them. This study emphasized the latter aspect because

several proteinases were found in pannus tissue although some of them seem to be more important than others. Cartilage and bone destruction in RA seems to result form invasion by pannus and to be directly mediated by proteinases produced by cells in pannus. The capability of rheumatoid fibroblasts to produce cartilage and bone degrading enzymes, as well as to stimulate osteoclast formation, suggests that fibroblast may be an important cell in pannus invasion and in hard tissue destruction. Therefore, another task in RA research is to characterize further the phenotype of these fibroblasts and to find the triggering effects that initially induce the aggressive behaviour of these cells.

The activity of proteolytic enzymes in the tissues is controlled at several steps, which include gene expression, protein production and secretion, activation of proenzymes and stabilization and/or inhibition of enzymes activities by natural inhibitors, e.g. TIMPs inhibiting MMPs; also inhibition of RANKL by OPG plays an important role. These molecules, especially various MMPs, have been intensively studied and many of them have been suggested to be involved in RA tissue destruction. For the past several years, both naturally occurring and synthetic inhibitors for MMPs have been studied as potential future therapeutic drugs, but many of these have failed in clinical trial for dose limiting toxicity and other adverse effects, and no compounds have been licensed for clinical use thus far. It has also become evident that some MMPs may actually exert anti-inflammatory effects. To overcome these challenges, it is necessary to understand the specific roles of various proteinases in more detail and then to design more selective inhibitors to target individual MMPs, perhaps for example MMP-3. However, the present work also suggests that inhibition of one single target may not be enough and that this target might be a moving target and differs during different stages of arthritis. Locally produced disease markers, like MMPs, could also be developed to prognostic indicators of severity of the erosive process as well as to demonstrate the degree of inflammation and the stage of inflammatory destruction in joints stricken with RA.

None of the disease-modifying anti-rheumatic drugs (DMARDs) or biological agents or combinations thereof seems to be able to provide hundred percent long-term protections against joint destruction. TNF is a key regulatory cytokine in RA inflammation and has been shown to be a good therapeutic target. Accordingly, TNF blockers are the most widely used biologicals at present. Three anti-TNF biologicals have been registered in Finland. In combination with methotrexate (MTX) they diminish cartilage damage and bone destruction, probably in part by increasing OPG and thus reducing the effect of RANKL (Feldmann and

Maini 2008). However, serious adverse effects have been observed and some patients are non-responders or do not respond adequately. The trial and error approach, however, shows in retrospect that in most patients the benefits of anti-TNF treatment exceed the risks. Blockade of IL-1 has also been proved to be clinically beneficial (Konttinen L *et al.* 2006), but less so than TNF inhibitors, perhaps due to other cytokines, e.g. IL-17, overtaking the role of IL-1 (van den Berg *et al.* 2007). However, the combination of TNF and IL-1 completely blocked formation of bone erosion in a mouse model of arthritis, being more effective than monotherapies with one of these drugs alone (Zwerina *et al.* 2004). Blockade of IL-6 is a new therapeutic target in RA (Oldfield *et al.* 2009) and clinical trials with antibodies against other pro-inflammatory cytokines eg. IL-15 and IL-17 are in progress. Consideration, however, must also be paid to redundancy, because some cytokines have almost identical properties and the diversity makes it difficult to predict the additional value of the specific blockade of these cytokines.

The use of gene therapy, stem cells and tissue engineering, has opened up new possibilities in treating arthritic diseases. One promising strategy is the antisense therapy, such as ribozymes, oligodeoxynucleotides (ODNs) and small interfering RNA (siRNA), which has a unique ability to down-regulate or silence gene expression by turning down the production of pathogenic proteins and cytokines. Some promising results have been observed in animal studies by inhibition of NF-kappa B, cathepsin or MMP expression to reduce pathological signs in arthritic joints and a few ribozymes have qualified for clinical trials. Recently, endogeneous microRNAs (miRNAs) have been in focus as a therapeutic target because of their ability to regulate various physiological and pathological processes. Few pilot trials using miRNA treatment have already been reported, but it is very likely that more epigenetic research based on miRNA will emerge in the near future. Mesenchymal stem cells are also known as bone marrow stem cells and multipotent mesenchymal stromal cells (adult stem cells) isolated from adult tissues. They have the potential to differentiate into various cells, including chondrocytes, osteoblasts and adipocytes, in response to appropriate stimuli and could be used for cell therapy in arthritic diseases. The usage of autologous stem cells from a patient, which are given back to that same patient, avoids the host rejection responses. A very important feature of stem cells, especially in treatment of RA, is their potent immunosuppressive and anti-inflammatory effects. Direct injection of stem cells into damaged tissue has been shown to decrease cartilage destruction and some clinical trials are going on. In tissue engineering stem cells are used to build a functional 3D scaffold to replace

damages tissue, especially cartilage with poor inherent repair properties. Marrow stimulation techniques, where holes were drilled into subchondral bone, and autografts, where healthy cartilage replaces the damaged tissue, are used, but also the autologous chondrocyte implantation (ACI) method and later matrix-induced ACI have been introduced. Nowadays much attention is paid to use of autologous stem cells in different kind of biodegradable scaffolds.

The role of cathepsin K and RANKL in different bone diseases has inspired drug companies to produce specific inhibitors for the treatment of bone resorption and some of these pharmacological drugs have reached clinical trials. Various DMARDs and biologicals used in the treatment of RA, like methotrexate, sulfasalazine and infliximab, inhibit the expression of RANKL and increase the synthesis of OPG, a RANKL antagonist, in rheumatoid synovial fibroblasts (Lee *et al.* 2004). A promising tool for inhibition of erosive joint damage that results from osteoclast-mediated bone resorption is biological anti-RANKL antibody treatment, which has been shown in preliminary studies to decrease bone turnover and inhibit structural damage in patients with RA (Bekker *et al.* 2004, Cohen *et al.* 2008). Several studies have also demonstrated that OPG is a potent inhibitor of bone loss, which affects bone density and mass in mouse and humans (Bekker *et al.* 2001). Cathepsin K inhibitors have also successfully moved into clinical stages and have been found to inhibit collagen breakdown and to improve bone formation. The chemical structures of these selective inhibitors have not been revealed, but the available clinical data is encouraging and suggests that these inhibitors have potential to become novel disease-modifying drugs (Yasuda *et al.* 2005). Transgenic mice expressing catalytically inactive ADAM8 expressed decrease incidence and severity of inflammatory arthritis whithout the risk of immunocompromise (Zack *et al.* 2009). ADAM8 is quite a novel protein in osteoclast research area and the research for its role in different diseases is ongoing and this molecule serves attention also as a diagnostic and prognostic marker and as a potential target for treatment.

The articular protein network is very complex, especially from the point of view of selection of potential therapeutic targets. It has to be taken into consideration that expression patterns may differ between individuals and different disease stages and that parallel pathways exist so that the function of one can be overtaken by another. In principle, long-term administration may induce tolerance and reduce the efficacy, which may enforce to dose escalation, which

again may increase adverse effects. First and foremost, current drug therapies can provide remission and should be given early during the course of the disease, but they do not cure. Therefore, development of novel anti-RA drugs is still needed. RA is a very complex syndrome and it seems that it is difficult to induce remission by only one drug. Thus, instead of monotherapies, combination treatments are widely applied already using conventional anti-rheumatic drugs (Nordström *et al.* 2006). This may well apply also for current and future biological drugs, although at present the experiences of the use of biological drugs in combinations are not encouraging. Finally, it remains to be seen whether the drug combinations to be used should be tailored according to the stage of RA, e.g. in early vs. in advanced disease. The final goal is not only to alleviate symptoms but to prevent tissue damage, preserve function, induce clinical remission and – ultimately - to cure the patient.

The initiating and perpetuating antigens or agents of chronic inflammation are not known, therefore, no treatment specifically targeting them exist. It seems that anti-inflammatory treatment alone with NSAIDs, coxibs, glucocorticoids, DMARDs or biologicals is not sufficient and that is why more attention should be paid to the cause and mechanisms of the disease initiating it and finally leading to cartilage and bone destruction. Usually when RA is diagnosed, the underlying disease has already been ongoing for a while and cartilage damage may already have developed. If it would be possible to make the diagnosis, when only aggrecan has been partially degraded but the supporting collagen network is still intact, the normal state of the cartilage could still be restored. In any case, understanding of the mechanisms responsible for cartilage and bone destruction and pannus invasion may open still new modes of prevention and treatment of this destructive joint disease and diminish the need for endoprosthetic surgery.

9. ACKNOWLEDGEMENTS

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